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THE WORK OF DMITRIY NIKOLAYEVICH NASONOV

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A. S. Troshin

Dmitriy Nikolayevich Nasonov, outstanding Soviet scientist and creator of a leading school of cytology in the USSR, died two years ago.

Dmitriy Nikolayevich held the banner of Soviet cytology high and did much to advance it. We have a right to be proud of his achievements. Together with his many co-workers he collected a vast amount of important experimental data that served as a foundation for progressive new theories. It is often said that we lag behind foreign cytologists in several respects. However, there is no doubt that the field in which Dmitry Nikolayevich worked directly--cellular physiology--is one of the finest in the world. For this he is entitled to the major credit.

Dmitriy Nikolayevich's creativity is astonishing in its purposefulness. He made many brilliant investigations all closely interrelated, each emerging naturally from the preceding one. He never cared to go into secondary subjects, being attracted by the most significant and exciting problems. Whatever he engaged in, the result was something new and important.

Dmitriy Nikolayevich's early work dealt with cellular organoids and their functions. His first scientific paper, published in 1918 while he was still studying with the well-known Russian histologist Aleksandr Stanislavoch Dogel', was a report on the stretching spindle fibers of dividing plant cells. This excellent work was awarded a gold medal.

He then made a detailed study of the Golgi apparatus, demonstrating the connection between this cellular organoid and the secretory processes in the cell. He also showed that the pulsating vacuole of the protozoans is a homolog of the Golgi apparatus in the cells of multi-cellular animals. This work became widely known. In 1926 he obtained a Rockefeller grant enabling him to spend a year in the Wilson Laboratory of Columbia University and successfully continue his efforts to elucidate the function of the Golgi apparatus.

Early in the 1930's Dmitriy Nikolayevich showed that vital dyes penetrating into the cell appear in the form of granules near the Golgi apparatus, where granules of the secreta are normally found. The cytoplasm and nucleus generally remain unstained, or only the cytoplasm takes a weakly diffuse strain.

However, when the cell is subjected to asphyxiation, elevated temperature, acidosis, alkalosis, etc., the nature of the vital stain undergoes a marked change. Granules of dye do not form, while the cytoplasm and nucleus take an intensely diffuse stain. The differences in distribution of the vital stains made it possible to use intravital staining as an indicator of the functional state of the cells.

These observations led to an extensive series of studies which were accelerated in 1933 when Dmitriy Nikolayevich, at A. A. Zavarzin's suggestion, organized a laboratory of cytology in the general and comparative morphology division of the All-Union Institute of Experimental Medicine, and again in 1935 when he adopted A. A. Ukhtomskiy's suggestion to organize a laboratory of cellular physiology in the Physiological Institute, Leningrad University.

It was shown that the most varied cells always react to the same complex of tinctorial and colloidal changes when excited by a great variety of physical and chemical stimuli (applied in fixed doses). These changes in the protoplasm are reversible if the effect of the external stimulus does not go too far. Nasonov and Aleksandrov in 1934 called the set of reversible modifications in the protoplasm caused by injurious agents paranecrosis.

Extensive research conducted by Nasonov's school subsequently proved that paranecrosis is a process developing in phases like Vvedenskiy's parabiosis and that paranecrotic changes in cells constitute a firm foundation of the parabiotic process. Establishment of this fact was tremendously important for the further progress of our science since from that time on the two great Leningrad schools--Vvedenskiy-Ukhtomskiy and Nasonov--were able to form a united front in studying the basic physiological processes taking place at the cell level.

A detailed investigation of paranecrosis led to the creation of a theory according to which alterations of protoplasm proteins underlie paranecrotic changes in the cell. These alterations resemble the initial phases of denaturization of native proteins.

The reports analyzing the effect of stimuli on the cell were surveyed by Nasonov and Aleksandrov in their monograph Reaktsiya zhivogo veshchestva na vneshniye vozdeystviya. Denaturatsionnaya teoriya povrezhdeniya i razdrazheniya (The Reaction of Living Substances to External Actions. Denaturization Theory of Injury and Irritation) (1940), which won the Stalin Prize in 1943.

Elaboration of the denaturization theory brought Dmitriy Nikolayevich into conflict with the membrane theory, which he then re-examined in toto.

Dmitriy Nikolayevich and his co-workers made a thorough study of narcosis, cell permeability, bioelectric properties of cells, etc. Elucidation of these phenomena within the framework of the membrane theory encountered serious difficulties and contradicted a great many facts.

According to the protein (denaturization) theory of injury and irritation, paranecrotic changes in protoplasm underlie, at a certain depth, the reversible suppression of cellular functions, e.g., in cell narcosis. This theory holds that cell permeability and distribution of substances between the cell and its surroundings are due not to some hypothetical cell membrane, but to the protoplasm as a whole, its properties as a solvent, and the ability of cellular colloids to combine chemically and through absorption with substances entering the cell, i.e, sorption processes are based on cell permeability.

The bicelectric properties of cells too received new clarification within the framework of the protein theory. These properties are determined by the state of the mineral substances in the protoplasm and their redistribution after injury and excitation Injury and excitation cause a release of ions in the protoplasm, which is the reason that phase and diffusion potentials appear on the border between the intact portion of protoplasm and the injured or excited portion.

Paranecrosis was regarded at first chiefly as reversible injury of cells and it was simply assumed that the same complex of paranecrotic changes were also responsible for the physiologic excitation.

This assumption was fully corroborated by a series of reports published by Nasonov and his co-workers after the war. He was right in regarding paranecrosis as a reaction underlying both reversible injury and normal (physiologic) excitation.

The denaturization theory of excitation and injury, which reveals the leading role of protein in cell activity, is a major contribution to biology. It is helpful in clarifying many phenomena and it is stimulating further research into intimate aspects of the mechanisms that ensure cell activity.

Relying on the vast amount of factual data obtained from the intense work of numerous co-workers over many years, Dmitriy Nikolayevich concluded that local and spreading excitation are profoundly related and that a certain quantitative relationship always exists between the magnitude of cell reaction and the magnitude of the stimulus that provokes this reaction. These conclusions were in harmony with the ideas on the nature of excitation advanced by N. Ye. Vvedenskiy, creator of the theory of parabiosis.

This view of cell excitation was the basis of Dmitriy Nikolayevich's gradual theory of excitation, which he first formulated in 1948. The gradual theory defines the quantitative relationships between magnitude of irritation and magnitude of reaction and the resultant principles that govern the transition from one type of excitation to another as well as the mechanism by which excitation is transmitted.

The ten years elapsing since publication of the gradual theory have proved its soundness and Dmitriy Nikolayevich's scientific foresight. The gradual theory has been confirmed in every detail by many investigations involving different conducting structures (nerve, nerve fiber, muscle fiber). The consequences flowing from it have also been confirmed: decremental conduction of subliminal excitation, incremental momentum of the action potential, excess of the maximum reaction over the magnitude of the traveling impulse at the site of excitation, and presence of decremental conduction of the maximum local reaction to the level of the regulated impulse.

Formulation of the gradual theory of excitation is the peak of Dmitriy Nikolayevich's creative endeavors. It can be regarded as a major biological idea that holds broad promise for research on the principles and mechanisms underlying the reaction of living systems to external influences.

Dmitriy Nikolayevich and his co-workers later solved some other problems in cellular physiology. They concentrated, in particular, on irritability and the elaboration of methods to measure it. Here too Dmitriy Nikolayevich made many valuable new contributions both of theoretical and of practical nature.

Even this sketchy account of the main stages of Dmitriy Nikolayevich's work reveals him to be a scientist of broad vision and a genuine innovator. His original research goes beyond biology and is justly included among the more significant achievements of Soviet science.

The creative path trod by Dmitriy Nikolayevich found expression in his views on the problems and trrnds of cytology. He understood cytology in a profound sense as a branch of science in which morphologists, physiologists, and biochemists had to work out together the basic principles governing cell activity. Accordingly, he was entrusted by the Presidium of the Academy of Sciences USSR with the task of organizing the Institute of Cytology in Leningrad. The Institute of Cytology of the Academy of Sciences USSR began to function early in 1957 under his direction and to implement the program he laid out.

High devotion to scientific principles, purposefulness, capacity to make severe demands on himself and co-workers--these are the qualities that characterized Dmitriy Nikolayevich as a man and as a scientist whose legacy will require more than one generation of specialists to execute. His ideas and inspired creativity will always be a reliable support in their scientific quests.

SUBSTRATAL CHANGES IN PROTOPLASM FOLLOWING LOCAL AND SPREADING EXCITATION

(Paper read 13 May 1957 at a conference on the problems of N. Ye Vvedenskiy's parabiosis. It was reconstructed from the notes of A. V. Zhirmunskiy and I. P. Suzdal'skaya.)

/Pages 605-613

D. N Nasonov
Institute of Cytology, Academy of Sciences USSR, Leningrad/

Although I was not a student of N. Ye. Vvedenskiy and I began my career as a histologist, I nevertheless came to the same conclusions as the university school of physiology. Before the war our group concentrated largely on the processes that take place at the cell site where stimuli are applied. Since the work has been summarized in a monograph (Nasonov and Aleksandrov, 1940), I shall touch only briefly on the contents of the book, confining myself mostly to the research performed after its publication.

We discovered that when living protoplasm is acted upon by a great variety of chemical and physical stimuli--mechanical, thermic, radiation, audible sounds, high hydrostatic pressure, narcotics, acids, alkalis, and other injurious agents--a complex of similar reversible changes inevitably appear in the nucleus and cytoplasm. We called these changes paranecrosis.

N. Ye. Vvedenskiy (1901) showed that the nerve too is characterized by an identical nonspecific, physiological reaction to different external influences. He called this reaction parabiosis and assumed that it was typical not only of the nerve, but also of any living system. A comparison of our data with Vvedenskiy's led us to conclude that paranecrotic changes in protoplasm underlie parabiosis.

After studying the indications of parabiosis, we became convinced that the action of different agents on living cells causes reversible denaturization of their native proteins. The following considerations supported this view:

(1) With denaturization as with paranecrosis there is decreased dispersity of the proteins.

(2) Viscosity increases in both cases.

(3) A shift of the reaction toward the acid side is observed in both cases.

(4) Finally, in both the substrate has increased capacity to bind basic and acid stains.

The latter was originally known only with respect to living protoplasm. To convince ourselves that the reason for intensified sorption of stains by stimulated cells is denaturization of native proteins, we set up experiments involving the staining of protein solutions (blood serum, ovalbumin) in native state and denatured by various methods.

It turned out that the same agents which act as stimuli and intensify sorption of the stains by the living cells are responsible, when applied to solutions of native proteins, for their denaturization and strengthen their ability to bind basic and acid stains (Aleksandrov and Nasonov, 1939). We attributed this to the fact that the action of stimuli on protoplasm or denaturization of proteins in vitro brings about changes in the configuration of the protein molecules along with the appearance of ionized groups (SH-, OH-, etc.), thus causing increased binding of the stains. Denaturization with paranecrosis is reversible denaturization in the early stage. However, if the action is strong, the effect is impossible or difficult to reverse. This circumstance, which is important in judging the chemical nature of the excitatiin, was subsequently confirmed on pure protein preparations in our laboratory by A. D. Braun (1948a, b), and by Haurowitz, Di-Moia, and Tekman (1952).

The sorption properties of denaturized proteins are intensified and not only with respect to stains. Fischer (1935) showed that chicken ovalbumin denatured by high temperature intensifies the binding of heparin. Studying the binding of labeled methionine by native proteins, A. G. Pasynskiy, M. S. Volkova, and V. P. Blokhina (1955) and T. Ye. Pavlovskaya, M. S. Volkova, and A. G. Pasynskiy (1955) found that these proteins had increased sorption properties when denatured in various ways.

In the opinion of many modern biochemists, which we share, the ionized groups in native molecules that appear in protein molecules after denaturization are inverted within the protein globule and cannot react with macromolecular electrolytes, especially with stains. Denaturization disrupts the configuration of the protein molecules, which results in the appearance on their surface of ionized groups that can bind the stains found in the solution (cf. Haurowitz, 1936).

A serious objection to the denaturization theory of stimulation was the prevalent view of denaturization as being irreversible in principle. However, a series of investigations proved that in the initial stages of the process denaturization of proteins was definitely reversible, sometimes heterodromically, but more often homodromically (Anson and Mirsky, 1934; Kunitz, 1948; Belitser, 1950).

After the war our group made further studies of local excitation and the correlation between local and spreading excitation. In a number of papers dealing with the action of various agents on muscles, it was shown that contractures, paranecrosis, and refractoriness (narcosis) of muscular tissue arise more or less simultaneously and that parabiosis and paranecrosis represent, therefore, different aspects of the same phenomenon--the reaction of muscles to external stimuli (Nasonov and Ravdonik, 1947; Nasonov and Suzdal'skaya, 1948; Nasonov, 1948, 1949; Butkevich, 1948; and others). Our views were opposed on the terminological grounds that the concept of "reaction" implies "reverse action" while the stimulus causes, as it were, a purely passive change in the tissue, its destruction. However this objection is unfounded. By parabiosis and paranecrosis we understand reversible changes, which, of course, include "reverse action," i.e., "reaction." Recent papers by N. S. Panteleyeva (1953), M. N. Kondrash (1954), V. S. Misheneva (1955) and others cite biochemical data that confirm our view.

We assume on the basis of some general considerations relating to the evolution of excitation that a reparative reaction was also the primary reaction from which the excitation subsequently developed. One may well believe that the most primitive of organisms -- blobs of protoplasm -- had to possess the ability to restore those injuries to their structure which inevitably arose after abrupt changes in the external environment. Indeed, it is inconceivable that the organisms could have existed without such ability. This property of a living system is apparently as old and as fundamental as the capacity for metabolism, division, and growth. According to this point of view, reversible injuries of protoplasm are to be considered the most primitive form of excitation.

If the ability to repair "damage" was the first stage in the evolution of excitation -- the "damage" being evidently the impetus to repair -- the next stage had to be the ability not only to "fix" the injury, but also somehow to guard against or escape from the irritant. The injury necessarily became the trigger mechanism both for the biochemical processes leading to repair and for the processes resulting in protection against or avoidance of danger.

Finally, as animals increased in size with the development of multicellular structure and nerve tissue, the most complete form of excitation evolved, i.e., spreading excitation, which serves to transmit signals swiftly from one part of the organism to another.

We were at first inclined to regard paranecrotic phenomena in the cells chiefly as "reversible injury," but we later concluded that local excitation arising at the point of application of a stimulus is caused by reversible denaturization of proteins and that similar changes take place even when excitation is conducted through the fibers. This view is supported by observations revealing that paranecrotic changes in protoplasm may spread slowly through the cell (Verworn, 1896; Chambers and Rényi, 1925; Chambers, 1925; Chakhotin, 1935; and others). After carefully studying the phenomenon of spreading injury in muscle fiber (so-called Zenker's degeneration), we became convinced that it is produced by means of an electric mechanism similar to the way excitation is transmitted along conducting fibers (Rozental', 1946; Nasonov and Rozental', 1947; Aleksandrov, 1948). This work was recently repeated in Western Germany by Rotschuh (1955), who reached the same conclusions that we did, although he was unaware of our research.

We then set out to investigate spreading excitation, but again encountered the membrane theory of permeability the soundness of which we had long doubted chiefly as a result of our efforts to check the applicability of osmotic laws to changes in the size of cells in media with different osmotic pressure (Nasonov and Ayzenberg, 1937; Nasonov, 1938; Ayzenberg, 1939).

A critical re-examination of the membrane theory of permeability led us to reject it <u>in toto</u>. This re-examination was facilitated by the conclusion reached by many foreign scientists that the theory did not square

with the facts obtained by new methods, particularly those using radioactive isotopes. Current hypotheses of a "pump" and "active transfer" advanced by some foreign investigators are speculative superstructures on the membrane theory and lack direct factual support.

In its place has been advanced the sorption theory of cell permeability and bioelectric phenomena expounded by A.S. Troshin in a monograph (1956), which is now available in a German translation published by G. Fischer in Jena. (A. S. Troshin's monograph was published in the German Democratic Republic in 1958 - Ed.).

There is every reason for believing that protoplasm is a conservative system whose water possesses very low solvent properties as compared with the water of the environment. According to Troshin, penetration and accumulation of substances in cells are determined mainly by three factors:

1. Distribution of dissolved substances between protoplasm water and environment water.
2. Adsorption of substances on the protoplasm.
3. Chemical bonding of substances by protoplasm colloids.

Most cell electrolytes in a resting state are chemically bound by proteins. If damaged or acted upon by local or spreading excitation, the protein-electrolyte complex disintegrates, free ions appear, and bioelectric potentials arise.

It was conjectured that local excitation arising near the site of stimulation results in reversible alterations of cell proteins and that similar changes take place even in the case of spreading excitation. It was natural, therefore, to conceive of the possibility of finding increased protoplasm colorability also during the excitation caused by such stimuli as a physiologic mediator or an impulse traveling along the conducting fibers.

Experiments yielded positive results. Intensified colorability by basic and acid stains of nerve cells in frogs and various mammals was observed when nerve processes were stimulated by an electric current or mechanical trauma (Kotlyarevskaya and Boldyrev, 1939; Romanov, 1948 a, b, 1949, 1953 a; Smitten, 1949; Ushakov, 1949; Zarakovskiy and Levin, 1953; Lev and Rozental', 1958).

Stimulation of the sciatic nerve and stomach mechanoreceptors and reflex conditioning caused increased binding of stains in the cerebral cortex of rats and mice (Levin, 1952; Romanov, 1953 a, b). However, it was not possible to identify the histologic elements responsible for this reaction.

It has also been shown that there is increased sorption of methylene blue by motor endings in frog muscle following electric stimulation of the nerves (Shapiro, 1953; Chetverikov, 1953).

Increased sorption of basic and acid stains by tonic and tetanic frog muscles was noted both when the muscles were treated with acetylcholine and when the nerves were stimulated with an induction current (Kiro, 1948; Vereshchagin, 1949).

Finally, increased binding of neutral red was observed after reflex stimulation of the pancreas of mice (Nasonov and Suzdal'skaya, 1953) and after repercussion changes in the cornea of rats (Zhinkin and Korsakova, 1951; Zhirmunskiy, 1955).

Intensified colorability of excited nerves was first described in nonmedullated nerves of invertebrates. N. V. Golovina (1955) found increased sorption both of basic and of acid stains after electric stimulation of the cerebrovisceral connective of the fresh-water mussel. Stimulation of this nerve is characterized by decremental conduction and a long-drawn-out regenerative phase (Zhukov, 1946, and others). It must have been easy, therefore, to record substantial changes in these specimens, for Golovina discovered that the decrement of nerve stain corresponded to the decrement of conduction. Segments of the nerve lying closer to the stimulating electrodes stained more intensely than the segments further away.

Intensification of the stain in another nonmedullated nerve -- that of the crab -- was observed by B. P. Ushakov (1950). The crab's nerve resembles the nerve of vertebrates in many physiological respects, but differs principally in duration of the regenerative processes (Zhukov, 1939). In Ushakov's experiments statistically valid intensification of nerve colorability occurred only with a stimulation frequency of about 80 to 90 cycles.

The initial attempts to find increased vital staining in the medullated frog nerve were not successful (Smitten, 1949). The reason was either the presence of myelin, which could have prevented penetration of the stain, or the great speed with which total regeneration of the resting nerve fiber takes place in the medullated nerve due to the transitoriness and wave-like character of spreading excitation. However, methods were eventually devised to increase binding of the stain when the nerve was excited. The amount of stain absorbed varied with the frequency of stimulation, the maximum being about 100 cycles (Nasonov and Suzdal'skaya, 1957)

These data on the binding of vital stains by various tissues are in themselves sufficiently indicative of denaturization changes resulting from physiologic excitation. Several recent reports provide additional corroboration. For example, Ye. V. Kornakova, G. M. Frank, and D N. Shteyngauz (1947) found a slow increase in viscosity of the frog sciatic nerve when irritated electrically. Flaig (1947) observed a decreased rate of axoplasm outflow from the cut nerve fiber of the cuttlefish following stimulation, which undoubtedly also suggests increased viscosity. Some papers describe reduced opacity in stimulated nerve (Hill and Kaynes, 1949; Hill, 1950; Tobias and Solomon, 1950: Lyudkovskaya, 1952; Lyudkovskaya and Frank, 1952), which indicates diminished dispersity of protoplasm colloids. Finally, the American scientists Ungar, Ashheim, Psychoyos, and Romano (1957) noted that proteins isolated from stimulated frog and rat nerves and from the

cerebral cortex of stimulated cats exhibit the same shifts in the ultraviolet absorption spectrum as do denatured proteins isolated from the nerves and brain at rest. All these data reveal that paranecrotic changes take place in conducting tissues as residual shifts after the passage of waves of spreading excitation.

We maintain that reversible protein reactions, which resemble the denaturization of native proteins, underlie not only local, but also spreading excitation. There are grounds for regarding these reactions, and not increased permeability of the border membranes as had been assumed hitherto, as primary in the process of cellular excitation. Paranecrotic changes arise after the action of an irritant, which is a mechanism triggering biochemical transformation. The biochemical papers of Deuticke and Ebbecke (1937), V. S. Misheneva (1955), and others support this view.

The term "parabiosis" might have been used instead of "paranecrosis" A A. Ukhtomskiy advised against it since parabiosis was studied in conducting tissues, whereas paranecrosis was observed in all tissues. However, there is no difference in theory between the two terms.

Parabiosis induced in a nerve is a model, but is nevertheless excitation. What are the forms of excitation in an organism that can be identified with parabiosis? The activity of adenoblasts, for example, may be so identified. It is highly probable that excitation of the smooth muscles, excitation after tissue inflammation, when the connective tissue cells are mobilized, are other examples of persistent excitation. I consider it likely that excitation in terms of evolution, as mentioned above, was an injury capable of being repaired, and I am collecting data that point to the relationship of these phenomena. In subliminal manifestations in the nerve we have something very close to parabiosis, but these phenomena spread. Excitation of a nerve, muscle, nerve ending, ganglion, or cortex is marked by residual changes resembling paranecrosis.

Excitation is frequently taken to mean purely the first phase of the parabiotic process while parabiosis is a state akin to the death of tissue. It seems to me that we should use parabiosis, local excitation, to designate not a state, but an entire process -- the full set of changes -- taking place from the time a stimulus acts until the tissue dies. This ties in more easily with the views on spreading excitation. After all we are not pulling away the absolute, refractory phase from the traveling wave. Thus, by parabiosis following local excitation we must understand an entire process.

Summary

When living protoplasm is acted upon by various types of physical and chemical stimuli, a set of reversible or paranecrotic changes arise. A comparison of the nonspecificity or homogeneity of these changes with the nonspecificity of the functional parabiotic changes following cellular stimulation reveals that parabiosis and paranecrosis are different ways of describing the local reaction of a living system to action from without

and constitute the external expression of one of the fundamental properties of living matter -- irritability. This has been confirmed by research on muscular tissue showing that contracture, refractoriness (narcosis), and paranecrosis develop more or less simultaneously.

The paranecrotic and, consequently, parabiotic reaction of living protoplasm is based, in the author's opinion, on reversible changes in its proteins that resemble the initial phases of denaturization changes in native proteins.

Paranecrotic changes characteristic of local excitation (change in viscosity, intensified colorability, decreased dispersity of tissue colloids) are also associated with the transmission of excitation through conducting tissues. Comparable biochemical reactions arise both in local and in spreading excitation -- relative, then absolute, refractoriness, electronegativity, and, in the case of muscular tissue, a contractile reaction. These facts confirm the view that there are no differences in principle between the local excitation of cells that arises near the application site of any stimulus and the excitation that spreads through the conducting fibers.

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AN INVESTIGATION OF THE LOCALIZED ACTION OF ULTRAVIOLET RAYS ON LIVING CELLS BY THE MICROBEAM METHOD

[Pages 614-626]

S. S Chakhotin
Institute of Cytology, Academy of Sciences USSR,
Leningrad

I have been working since 1909 in the new field of experimental cytology, the foundation of all modern biology and chemistry. My main efforts have been devoted to photomicrographic surgery. This field is a synthesis of the ideas of the German pathologist Rudolf Virchow on the cell as the basic laboratory of life -- expressed more than a century ago in his famous Cellular Pathology (1858) -- and the ideas of the French physiologist Claude Bernard, according to which the main task of physiologic research is to devise experimental methods such as enabled the physicists and chemists to achieve great success in the 19th Century (Bernard, 1878).

After studying the works of these invetigators, I concluded that experimentation on individual, isolated cells was essential. This approach to the study of cellular phenomena, to be sure, was not unknown before, but it was comparatively rare. I need only mention the names of Chabry (1887), Balbiani (1888), Maupas (1888), Verworn (1889), Wilhelm Roux (1895), Bataillon (1910), and Spemann (1918). However, in most instances the cells examined were fairly large (e.g., the eggs of frogs and other amphibia) and success depended on the manual dexterity and proficiency of the experimenter. The main difficulty encountered with isolated cells was the minuteness of most of them, which handicapped the investigation.

Later, after it had become increasingly clear that almost all the major biological problems entail a knowledge of the most intimate cellular processes, some investigators tried to circumvent the difficulty by using what I call cellular macroexperiments They are still used in cellular physiology and biochemistry. Routine physiological and chemical methods are applied to homogeneous masses of cells (bacteria, spermatozoa, ova, yeasts, unicellular organisms, blood corpuscles, etc.), the results and conclusions being extrapolated to the individual cell. Such, for example, were the well-known experiments of Warburg (1910a) and those of his students and followers on respiration in the sea urchin egg, then his investigation of glycolysis of blood elements (Warburg, 1910b) and cancerous tumors (Warburg, 1923), etc., or the work of Driesch (1891), Herbst (1893), and others on experimental embryology, and mechanics of development of the sea urchin egg, etc. A very common method now in vogue is differential centrifugation whereby the cells are destroyed while their contents are arranged in layers due to different specific weights. It is used to analyze various fractions of the homogenate where the chemical substances or organoids of the cells settle (nuclei, mitochondria, microsomes, etc.).

Although these methods are often useful, particularly in determining the chemical composition of cellular material, products of cellular activity, and (in connection with electron microscopy) ultrastructures of cellular organoids, nevertheless the results are of generalized character and cannot be substituted for actual microexperiments, i e , experiments on individual cells using microsurgical instruments.

My initial effort was to devise a mechanical micromanipulator, which I called a microoperator (Figure 1; Chakhotin, 1912a). This was followed by the apparatus of Chambers (1918), Peterfi (1924), de Fonbrune (1932), and others. However, I soon realized that mechanical operations, even when performed with extremely fine instruments, frequently do considerable damage because the act of penetration ruptures the surface of the cell, resulting in its death. Instead of a mechanical microneedle I tried to use photochemical action on living cellular substance in certain areas of spectrally decomposed light (the bactericidal effect of ultraviolet rays was already known). In 1912 I worked out a technique that I called the radiation microbeam method (Chakhotin, 1912b) and constructed an apparatus in which the "scalpel" is an exceedingly fine bundle of ultraviolet rays beamed at individual parts of the cell to limit surgical action to the organoids (Chakhotin, 1935a).

This apparatus (Figure 2) utilizes a certain monochromatic part of the spectrum, that is, the intensive and biologically highly active group of lines obtained from an arc between magnesium or cadmium electrodes -- 280 mu. (This source of ultraviolet rays was used by Köhler in his method of ultraviolet microphotography.) Quartz monochromators with Rohr background-lenses optically corrected for this group of spectral lines -- are employed to concentrate the rays in a microbeam. The light beams pass through a very narrow controlled slit whose ultraviolet reduced image is directed by the quartz objective placed upside down under the microscope stage to the object (cell) on a quartz plate that acts as a surgical bed. The microscopic image, therefore, serves as a pricking microbeam. It is focused in the dark by means of a fluorescent microscreen of very fine uranium glass particles 1 to 2u thick on the preparation alongside the cell while the position of the beam is fixed with the help of a needle in the eyepiece (Figure 3; Chakhotin, 1937a). This permits the micropuncture to be made in a light field for which the microbeam coming from the slit is passed through an opening in the mirror. Illumination on the side is provided by an ordinary microscope condenser whose light falls on the preparation through the same quartz lens as that used by the microbeam. When the preparation is moved by the micrometric screws of the microscope stage, the irradiated point of the cell is brought under the needle in the eyepiece. The cell is in a tiny drop of fluid covered with a layer of vaseline oil to prevent it from drying. The size of the image may be as large as 2 or 3u in diameter, but we were recently able to reduce it to 1 sq. u. Technically, therefore, it is even possible to irradiate parts of chromosomes.

Individual cells served as experimental material: protozoans (Chakhotin, 1935e, 1936), filar algae, ovicells, especially of sea urchins and Pholas (Chakhotin, 1920, 1938d, etc.), red and white blood corpuscles, large bacteria, spermatozoa, and isolated cells of multicellular organisms.

The indispensable conditions for successful employment of this method are availability of suitable microinstruments, possibility of selective irradiation of parts of a cell, i.e., protection of the other parts against the rays, and a simple method of postoperative observation of cells in the so-called "microclinic" (Figure 4; Chakhotin, 1937b, c, 1938a). The latter is used as follows. A drop of vaseline oil is deposited in the depression of a slide; droplets of the fluid into which the cells are transferred after the operation are placed with a tiny micropipette under the oil on the bottom of the depression. The cells live for weeks and even months in these drop chambers where they may multiply. At any time they can be observed, fed, and exposed to anything, particularly chemical substances, transferred to other drops, operated on again, etc The technical objective of all these methods and devices -- the possibility of rapid, reliable, and accurate work -- is thus attained. All manipulations of the individual cell, including irradiation and transfer to the microclinic, take about two minutes. A single cell, e.g., paramecium, amoeba, or microscopic ovum, can be operated on about ten times together with transfer to the "surgical bed" and then to the microclinic and back.

Microphoto surgical possibilities include: localized lesions and destruction of structures of cell organoids (Figure 5) and localized stimulation of their activity; enucleation; sticking two cells together, e.g., the blastomeres of different ova; study of colloidal changes in the cytoplasm or nucleus by combining the microbeam method with observations in a dark field (Chakhotin, 1935, 1938b); study of local permeability of a cell surface to different chemical substances, etc.

We present now some illustrations of successful employment of the ultraviolet microbeam method for various biological problems, e.g., parthenogenetic activation of ova of the sea urching (Figure 6) and Pholas. After the surface of the virgin ovum has been pierced at one point by the beam (Chakhotin, 1929), the egg membrane forms at the site and stretches over the entire surface of the ovum as in normal fertilization. As a result the ovum begins to divide and develop without participation of the spermatozoids.

Another example is teratological injury to individual blastomeres or their nuclei (Figure 7) in the developing embryo (Chakhotin, 1920). After the nucleus of one of the two blastomeres is pierced by the beam, cell division and development of the intact blastomere proceed normally and produce a branching of cells; development of the blastomere with irradiated nucleus, however, halts at the single cell stage, undergoing cytolysis after a time like any cell in which the metabolic processes (respiration, glycolysis) continue, but the resultant energy is not utilized for fission.

Other experiments revealed regeneration of the cell organoids injured by a microbeam. Cilia fall out on the surface of a paramecium at the irradiated site (Figure 8), but new cilia soon appear in their place in the cells put in the microclinic and swimming about freely; following division of the cell in two, no traces of irradiation remain on the irradiated part of the surface (Chakhotin, 1936a).

Another example -- an operation to blind the stigma of the flagellated euglena and prove that the stigma is a cellular eye (Chakhotin, 1936b). The experiment involved placing an euglena with a stigma at the base of the flagellum (a red speck) into a quartz capillary darkened at both ends in the microscope. The euglena normally moves to the edge of the shadow, makes a 180° turn, and goes back up to the shadow at the other end of the capillary. While moving the stigma is irradiated with a microbeam, after which the euglena enters the shadow.

Another experiment -- conditioning (reflex type) of a paramecium (Chakhotin, 1938c). A paramecium is placed in a tiny drop of water under the microscope. It moves continuously along the periphery of the drop. A vertical ultraviolet barrier is set up at a place in its path; it swims up, recoils, receives the beam, and again continues along the periphery. This is repeated many times; after 10 minutes it "learns" to get around the barrier, i.e., it goes around the drop off center. The barrier is removed, but the paramecium continues to swim in its new path -- a conditioned reaction has been formed, according to Pavlov, just as in the higher animals and man. After a quarter of an hour the reaction disappears, the "memory" of the dangerous place has faded, and the paramecium again encircles the drop on the periphery.

Another experiment makes it possible to measure the difference in absorption of ultraviolet beams between the cytoplasm and the nucleus (Chakhotin, 1935a). The microbeam passes into a microcompressor containing a cell, continues through the nucleus, and emerges from the preparation. It is reflected by the quartz prism under the compressor toward a photoelectric ell, where the deviation of the arrow is noted on the galvanometer scale (Figure 9). After the preparation is slightly shifted by the micrometer screws, the bean passes only through the cytoplasm and the arrow moves to another scale division. The quantitative difference in absorption of rays in both cases characterizes the chemical composition of the different parts of the cell and the changes resulting from influences to which the nucleus and cytoplasm may be subjected.

This method enabled us to experiment with local changes in permeability of the cell surface. Irradiating a spot on a sea urchin ovum and then placing it in a hypertonic solution resulted in a slight invagination at the site irradiated (Figure 10), but a protuberance on the same site in the hypertonic fluid (Chakhotin, 1921a). This method permits quantitative analysis of the role of the nucleus in cell respiration. A sea urchin ovum is placed in a quartz capillary where the fluid is separated from a solution of potassium hydroxide by an air bubble (Chakhotin, 1935a, 1936c). The eyepiece of the microscope has a scale to measure the size of the bubble. The ovum absorbs oxygen from the

bubble and isolates the carbon dioxide absorbed by the potassium hydroxide. The bubble shrinks in proportion to the time elapsing from the start of the experiment. We can thus determine the rate of cell respiration. Irradiation of the nucleus of the ovicell shows that changes in the rate of respiration do not occur here, i.e., the nucleus is not involved in the respiratory processes.

I should like to describe still another microexperiment (Chakhotin, 1935c). One of the contractile vacuoles of a paramecium is irradiated with a microbeam, then a second vacuole (Figure 11); they cease to pulsate (i.e., separate water from the body) and increase in size. Placed in the microclinic, the cell distends and finally undergoes cytolysis. However, if before this the cell is again placed on the "surgical bed," i.e., in a drop on a quartz slide, and its cytostome then irradiated, the cilia of the latter, which introduces water into the body of the cell, cease to beat, water no longer enters, and the cell transferred to the microclinic recovers. The water, moreover, is drawn by diffusion through the surface of the cell, swelling of the bodydisappears, the vacuoles begin to pulsate again, and the paramecium resumes normal swimming in the microclinic. This phenomenon of cellular "dropsy" somewhat resembles edema in a uremic crisis in higher animals and man and the "water diet" treatment.

All these surgical possibilities depend on the specific action of ultraviolet waves on different morphological structures of the cell, i.e., ultimately on the chemism, physiocochemical properties of ultrastructures, and biochemical processes in the cell and its organoids.

Two main facts stand out here: (1) coagulation of denatured protein colloids, and (2) the differing sorption of ultraviolet rays of certain wave lengths by the various chemical substances constituting the cell organoids, with their subsequent photochemical disintegration. I performed a variety of experiments along both lines to elucidate the essence of the problem. Here are some of them. Following local irradiation of portions of amoeba cytoplasm in a dark field (Chakhotin, 1935a), localized coagulation of the protein colloids took place at the site of irradiation (Figure 12).

The opposite picture was observed when one of the two nuclei of Amphileptus (Figure 13) was irradiated (Chakhotin, 1936a). It became more transparent in a dark field, i.e., dispersity of the colloids therein increased (their hydration increased). To achieve selective irradiation and to prevent ultraviolet ray destruction of the protoplasmic layer through which it has to pass before reaching the nucleus, it is necessary to increase slightly the concentration of calcium ions in the medium, which here stabilize the protein colloids of the surface (Chakhotin, 1935a, 1937d).

Two experiments are particularly important. Local irradiation of one of the two blastomeres of a sea urchin ovicell in sea water to which lithium ions have been added will cause that blastomere to swell because lithium has penetrated it and affected the protein colloids. We can thus selectively poison parts of an embryo.

Another experiment. If we irradiate the periphery of one cell of the fungus Ascoidea rubescens (of the Hemiascomycetes) in an alkaline solution, the vacuum siphon in the cell disintegrates into spheres in which the Brownian movement of the thick mass of luminescent micelle can be clearly seen in a dark field. This process is reversible -- the siphon quickly regenerates (Chakhotin and Gavodan, 1936a, b). However, if the reaction of the medium is acid, irreversible coagulation of the contents of the vacuum takes place. The same phenomena may be observed without irradiation if the medium has ammonia ions or liposoluble organic bases, e.g., choline; on the other hand, there will be coagulation if liposoluble organic acids (e.g., acetic acid) are added to the medium.

A final, revealing experiment (Chakhotin, 1912c, 1921b). Lipoid granular inclusions of lecithal character in a sea urchin ovum are stained red with vital neutral red, whereas sea water that has an excess of hydroxl ions is stained yellow with the same dyes. If we irradiate locally the surface of a cell, we can see the gradual change of the red color within the cell to yellow in concentric circles beginning with the irradiation site. We conclude that the change is caused by hydroxl ions of the medium penetrating the cell at a place of unusual permeability. However, this also happens, although to a lesser extent and more slowly, after irradiation of a red-stained ovum in a solution with a neutral reaction. Here too the color changes into yellow, thus indicating the presence of hydroxyl ions inside the ovum. Apparently the lecithins disintegrate photochemically into their constituent parts, particularly into alkaline choline, which is gradually carried in the cytoplasm to the adjacent granules, causing in chain fashion cytolytic disintegration with the formation of choline. Lecithen irradiated with ultraviolet light in vitro is known to yield choline.

The results of all these experiments suggest that the action of ultraviolet rays may be largely due to changes in cellular permeability. Consequently, a study of permeability may well throw light on a host of related questions. On the mechanism of cell permeability there are two hypotheses that at first glance seem to be contradictory: (a) the older hypothesis of the existence of a semipermeable coating or membrane on the cell surface, and (b) the sorption hypothesis (Nasonov and Aleksandrov, 1943; Troshin, 1956; Nasonov, 1959), which relies chiefly on the conception of a coacervate structure of protoplasm (Bungenberg de Jong, 1932). However, it is quite possible, indeed probable, that both explanations are valid and that the two factors coexist, with one or the other type predominating in a given entity. The physicochemical view of phases maintains that the surface layer of cytoplasm must be different from and denser than its inner layers; therefore, the surface film consisting of protein and lipoprotein molecules and micelle may have a coacervate structure with all the properties thereof.

However, there is still another possible view, one which does not contradict the sorption hypothesis, i.e., deplolarization of elements of the cell surface by ultraviolet rays (the cell surface is known to carry chiefly a negative electric charge). When irradiated, the surface is locally depolarized, causing a change in surface tension with certain mechanical consequences. Expansion or contraction of the pores in the surface layer then becomes possible. Finally, we can also have the action -- accelerating or inhibiting -- of ultraviolet light on ferments since catalytic phenomena are known to play a major role in most biochemical processes.

It follows from the above that there can be no single explanation, i.e., all the possibilities may coexist. The final answer awaits further research, which may consist of: (1) a combination of experiments using the microbeam method and chemical actions; (2) an investigation of changes in cellular reactions to irradiation under various temperature and pH conditions; (3) an investigation of surfaces of ultrastructures with an electron microscope; (4) an investigation of cellular reactions to microbeams of different wave lengths, particularly those of the infrared areas of the spectrum, and of the visible rays with simultaneous sensitization of objects by photodynamic substances in the spirit of Tappeiner's work (1900, 1911); (5) an attempt to use ionizing radiation for local micropunctures; (6) experiments with molecules labeled with radioactive isotopes and their detection in order to establish their topography in the cell.

The method that we first proposed in 1912 for studying cellular morphology and physiology with an ultraviolet microbeam was eventually employed by researchers in various countries. For example, in Germany Schleip (1929) and his students, Penners and others (1924) on the mechanics of ovum development in Tubifex and other worms, Seidel (1926) on insect ova, Dürcken (1933) on the ova of amphibia (Dürcken slightly modified my apparatus, adapting it to irradiation of larger and nontransparent objects -- the ultraviolet microbeam is aimed at them from above); in Italy Terni (1933) and his co-workers Arslan and Cojazzi (1934) on spermatozoids and epithelial cells; in Belgium Pasteels (1938) on the ova of molluscs, etc. More recently Zirkle, Bloom and Uretz (1953, 1955, 1956, and 1957) in the United States have conducted interesting research involving irradiation of parts of mitotic figures in triton (Triturus) cells, etc., using the ultraviolet microbeam method and ionizing radiation.

Summary

Microphoto surgery as a field of experimental cytology employing ultraviolet (UV) rays arose from a synthesis of the ideas of Rudolf Virchow and Claude Bernard. Its main feature, unlike cellular macroexperiments with homogeneous masses of cells, is microexperimentation on parts of single, isolated cells.

This article described the principle underlying an apparatus for micropuncture utilizing a monochromatic beam of UV rays of 280 mu, the diameter of which can be reduced to lu. The main prerequisite for success in the experiments is a "microclinic" where the cells operated on can be kept alive for a long period of time.

Examples of successful microexperiments were cited: parthenogenesis of a sea urchin ovum by focusing a local microbeam on the surface; teratological operations of individual blastomeres and their nuclei in the course of development; local coagulation of proteins in amoeba protoplasm at the site irradiated by the UV microbeam; loss of cilia at the irradiated site on the surface of Ciliata; invagination of the surface or purely local ruptures with outflow of some cytoplasm; irradiation of the stigma of euglena resulting in its blinding; formation of a conditioned reflex in a paramecium as shown by avoidance of the place in the medium where the UV microbeam is directed; local changes in the permeability of a sea urchin ovum at the site of an UV microbeam and penetration thereof by ions; investigation of the role of the nucleus in cell respiration by combining the capillary micromanometer and UV microbeam methods; halting of contraction in the vacuoles of a paramecium causing destructive swelling of the body of the cell, which disappears after beating of the cilia in the cytostome is stopped by irradiating the latter with a UV microbeam; using a photoelectric cell to measure the difference in absorption of a UV microbeam after local irradiation of points in the nucleus and cytoplasm.

All these microoperations are feasible because of the specific action of UV rays on different cellular structures and biochemical processes in the cell. This action is manifested in the denaturization of protein colloids caused by differences in the sorption of UV rays by parts of the cell and the subsequent photochemical disintegration of substances in these parts. It is sometimes necessary to stabilize the colloids on the surface layer of the cell by adding calcium ions to the medium in order to differentiate the effect of the microbeam on the nucleus and cytoplasm. Local changes in permeability make it possible to carry out elective poisoning of individual cells in a germ by various chemical substances.

The mechanism of local UV microbeam action on permeability may be explained by the sorption hypothesis, which assumes a coacervative structure of the cell. Sometimes, however, even thicker films may be found on its surface, and these are subject to the laws formulated by the membrane theory.

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PROTEIN STRUCTURE AND ION ADSORPTION IN THE MECHANISM OF CELLULAR EXCITATION

[Pages 627-640]

D. Ungar
Laboratory of Pharmacology, Vitamin Corporation of America, New York

Excitation is a fundamental process that links living matter to the environment. Excitation, recognized as a fundamental biological process by Glisson back in the 17th Century, was identified before the discovery of bioelectric phenomena by Du Bois-Reymond with motility, which is still the most reliable criterion of excitation. At the turn of the century Augustus Waller demonstrated that the excitation of living matter was a universal process.

Great progress was made in investigating the physiological phenomena associated with excitation, especially since the cathode oscillograph came into use. The chemical aspect, on the other hand, was ignored until comparatively recently.

For a long time we only knew about such chemical changes occurring in the cell during excitation as potassium loss and sodium gain. However, even these changes, according to the widespread theory of Bernstein (1902), resulted from mechanical factors, e.g., permeability of the cellular membrane. It is only now that we are becoming aware of the biochemical phenomena accompanying excitation. Most of the observations bear on the metabolism of an excited cell as compared with a resting cell. These facts are tremendously important in determining the sources of energy required for the process of excitation, but they affect the mechanism of excitation only indirectly.

An attempt is made in this paper to analyze some of the biochemical and physicochemical factors directly involved in the transition of a cell from a resting into an active state. The role of proteins in this process is examined in detail.

Several earlier investigations indicate that proteins are important in excitation. Intensified protein disintegration during excitation was observed by Soula (1913) and Hirschberg and Winterstein (1919). Intensification of protein metabolism in nerve centers following prolonged irritation was recently demonstrated by the microspectrophotometric observations of Hyden (1955) and the studies of Friedberg, Tarver, and Greenberg (1948), Gaitonde and Richter (1955), and Palladin (1956) of labeled amino acid incorporation. Geiger, Yamasaki, and Lyons (1956) and Ungar, Aschheim, Psychoyos, and Romano (1957) observed the formation of protein disintegration products during cellular excitation.

Evidence of more delicate changes in proteins can be found in the early works of Lugaro (1895) and Carlosson (1902-1903) on the affinity of nerve cells for certain stains. Nasonov and his co-workers (Nasonov, 1949, 1959; Nasonov and Suzdal'skaya, 1957; Troshin, 1956) were the first

to realize the true significance of these changes. After extensive research they showed that irritation of the nerve centers, myelinated and nonmedullated nerves, and muscles causes a variety of manifestations grouped under the common name of "paranecrosis." These include colloidal changes, increased viscosity, and heightened affinity for certain kinds of vital stains. They were interpreted as evidence that proteins in the excited cell undergo structural changes similar to those occurring in denaturization.

In 1955 when our work on tissue reaction to injury logically led us to the problem of excitation we were still unaware of Nasonov's research (Ungar and Damgaard, 1954, 1955; Ungar, 1956; Ungar and Kadis, 1959). We were convinced that this reaction is invariably accompanied by the formation of protease and subsequent proteolysis. However, it turned out that physiological irritation requires finer and more easily reversible processes than proteolysis. We therefore advanced the working hypothesis that normal excitation is marked by structural changes in proteins without rupture of the peptide bonds.

The existence of these phenomena was conjectured by Rapkine (1931), who observed them during egg fertilization, and by Mirsky (1936) in connection with the effect of light on visual purple.

The great progress in protein chemistry made during the last few years has considerably lightened our task and we no longer have to contend with the theoretical and technical difficulties that confronted the early investigators.

Changes in Configuration of the Protein Molecule

Denaturization of proteins is now regarded as a weakening of the solid structure of the molecule due to rupture of some of the crosslinks without hydrolysis of the peptide bonds. These crosslinks may be hydrogen bonds, salt bonds, bisulfide and phosphate bridges, thio ether bonds, or Van der Waals forces. When these bonds are broken, the protein spiral unwinds and forms irregular balls.

It was known back in 1933 (Anson and Mirsky) that denaturization may be a readily reversible process, but only a few -- Nasonov, Rapkine, and Mirsky -- understood the possible physiologic significance of the native protein $\rightleftarrows$ denatured protein reaction. Mirsky's assumption that light irritation causes changes resembling denaturization in the opsonic part of visual purple was confirmed much later by Wald and Brown (1951 - 1952).

There are many criteria of denaturization. Changes in solubility and viscosity have been extensively studied. Scientists are now using more up-to-date research tools. Infrared spectrophotometry (Ambrose and Elliot, 1951) is of limited value because water vigorously absorbs light in the same part of the spectrum as the groups affected by denaturization. The most promising method of determining which part of the protein molecule is spiralized and which part is unwound seems to be

that employed by Yang and Doty (1957) when they investigated the dispersion of scattered polarized rays. Another interesting approach is to measure hydrogen-deuterium exchange (Linderstrøm-Lang, 1955), which takes place more slowly in native than in denatured proteins.

Unfortunately, these methods are feasible only in investigating pure proteins. To conform our hypothesis, we had to employ methods applicable to tissue extracts. The technique of uncovering reactive side groups proved to be the most convenient. It has long been known that the number of side groups of amino acids (-COOH, $-NH_2$, = NH, -SH, -OH, etc.), which can be determined in various ways, grows in denatured proteins.

Our initial method was based on changes in the ultraviolet spectrum of proteins caused by uncovering the phenol hydroxyls of tyrosine (Crammer and Neuberger, 1943). A detailed account of this work was published in 1957. After studying isolated frog and rat sciatic nerves irritated in vitro as well as the cerebral cortex of cats, dogs, and rats, we concluded that the excitation process is accompanied by an increase in the number of ionized side groups of cellular proteins. This change was found to be reversible after irritation was halted.

Table 1

Changes in the Content of SH-Groups in the Rat Brain After Irritation*

Duration of irritation (in min.)	Time of regeneration (in min.)	-SH in ug	±Standard deviation	P - statistical probability	n- number of experiments
At rest		4.56	0.57	-	29
1	--	5.08	0.78	> 0.05	7
1	1	4.75	0.83	--	6
1	2	4.15	0.56	--	6
20	--	6.55	1.02	< 0.001	26
20	10	5.20	0.96	--	6
20	20	4.17	0.32	--	6

*Brain extracts were prepared in 0.15 M Nacl.

Spectrophotometry also proved to be useful in uncovering SH-Groups of cysteine (Benesh and Benesh, 1955; Ungar, Aschheim, and others, 1957). These could be measured much more accurately in other ways, especially by amperometric titration according to the method of Benesh, Lardy, and Benesh (1955). Table 1 cites the results obtained by this method for the cerebral cortex irritated through the central ending of the sciatic nerve. It shows that excitation increases the number of SH-groups titrated. The phenomenon appears after one minute of irritation, but it is more pronounced after 20 minutes (Ungar and Romano, 1958).

Similar results were obtained from experiments on nerve formations and on the submaxillary gland of dogs electrically excited through the chorda tympani, the urinary bladder of guinea pigs with direct electric excitation, and isolated dog retina excited by light. The same results were observed in experiments on nerves and on the brain, thus indicating that the phenomena have a common cellular nature.

The experiments performed by Dr. Kadis in our laboratory on euglena cultures show that light applied to these unicellular organisms also causes changes in the side groups of protein. His findings reveal that light acting on a system bound with chlorophyll causes structural changes in the proteins.

Table 2

Changes in the Potassium and Sodium Content, Rest Potential, and Free SH-Groups of Isolated Rat Diaphragm after the Action of Insulin*

Indicator	Control	Action of Insulin	Change (in %)	Authors
Potassium (in ug)	82.3 ± 32.8	130.4 ± 33.5	+ 58.4	Creese, D'Silva and Northover, 1958
Sodium (in ug)	53.0 ± 4.5	42.6 ± 4.5	- 24.4	The same
Rest potential (in mv)	70.0 ± 10,1	75.4 ± 9.0	+ 7.7	Zierler, 1957
-SH (in ug)	5.32 ± 1.08	4.14 ± 0.84	- 28.5	Ungar and Kadis, 1959

*The mean values ± standard deviations are cited.

The histochemical studies of Fisher and Zeman (1959) show that excited cerebral cells (in picrotoxin spasm) have a greater affinity for stains than do cells in a resting state. This can be proved by tracing protein changes in living cells or in cells fixed by methods that do not alter the protein structure. Several laboratories are now actively searching for these methods.

These data have led us to conclude that changes in the configuration of protein molecules can be directly related to the mechanism of excitation. If this assumption is valid, the phenomena will have to be linked somehow to electric changes and to the shift in potassium and sodium ion content characteristic of the excited state.

The effect of insulin on muscle cells makes it possible to verify this correlation. It has been demonstrated that the addition of insulin to isolated preparations of rat diaphragm causes the cell to absorb potassium and lose sodium (Flückiger and Verzar, 1954; Creese and others, 1958) besides increasing the assimilation of glucose from the medium.

This is accompanied by intensification of the resting potential (Zierler, 1957). According to this hypothesis, these changes are necessarily associated with a decrease in the SH-groups. It is evident from Table 2 that this was precisely the result obtained. The addition of 0.1 unit of insulin per ml to the preparation substantially reduced the number of SH-groups in the muscle (Ungar and Kadis, 1959).

It is probable that changes in protein structure are casually related either to a change in cell "permeability" to sodium or potassium or to a change in the affinity of cytoplasm for these ions.

Adsorption of Ions

According to the widely held membrane theory of cell permeability, the nonequivalent distribution of K^+ and Na^+ ions between cells and in the intercellular fluid as well as the shift of these ions during excitation can be explained by the unusual qualities of cellular membrane permeability and by the presence of an ion pump, which in the resting state ejects Na^+ and absorbs K^+. Although we have no direct proof that these mechanisms actually function, the abundant observations and hypotheses of Hodgkin, Huxley, Keynes, and their co-workers (Hodgkin, 1957) made it possible to construct a coherent theory that aroused great interest among neurophysiologists, particularly in Great Britain and the United States. The permeability theory was greeted with somewhat less enthusiasm by general physiologists, who are inclined to regard excitation as a phenomenon not peculiar to nerve formations alone and who are accustomed to deal with more complex systems than the isolated nerve electrically irritated.

We have made attempts to check this theory critically (Ungar, 1957). In this paper we want to set forth certain observations according to which the distribution of ions is determined not by membrane permeability or by the activity of pumps, but by the selective affinity of the cell, especially its proteins, either for K^+ in a resting state or for Na^+ in an active state. This view was advanced by Ling (1952), which he called the "fixed charges" hypothesis and subsequently worked out in greater detail (Ling, 1957, 1958).

If this view is correct, it may be possible to prove that resting cell proteins bind more potassium than sodium, whereas the affinity is reversed in the active state. The possibility of preferential binding of K^+ or Na^+ was shown for ion exchange resins, but not for proteins. The binding of monovalent cations with proteins was difficult to investigate, and it is doubtful that they can be bound as firmly as bivalent or trivalent ions.

Our first experimental approach to the problem was to measure the diffusion of K^+ and Na^+ from rat brain extracts in distilled water through a cellophane membrane. Extracts were prepared from the brain frozen either in a resting state or after 20 minutes of electric irritation. Specimens were taken from the external solution every 15 minutes and the potassium

and sodium content determined by flame photometry. Checking about 3 hours after an equilibrium was achieved, we found no difference between extracts from a brain in the active state. However, observations have shown that there is a marked difference between the two types of extracts in the initial stages of diffusion.

Laying off on the axis of ordinates the logarithms of the ratio of diffusing ions per unit of time (r) and on the axis of abscissas the logarithms of time (t), we obtain the straight-line relationship

$$\lg r = a - d \lg t, \qquad (1)$$

where a is the intersection of the ordinates at 0 (lg 1 min.); d is the slope of the straight line, i.e., indicator of the change in rate of diffusion. After measurements in the KCl and NaCl solutions this value proves to be proportional to the diffusion coefficient.

Figure 1 shows this relationship for Na^+ and K^+ in experiments with extracts from a brain in a resting state and from a brain in an excited state. It is evident that the diffusion rate of Na^+ is much higher in the brain in the resting state. For K^+ the rate is higher when the brain is in an active state, but the difference is not as great.

The difference between the active and resting states is more pronounced if the measurements are made at an early stage. In some experiments we tried to measure the diffusion rate during the first 5 minutes. The experiments here were set up somewhat differently: the external solution washed the cellophane membrane at a constant rate (about 1 ml/min.). Table 3 presents the results of these experiments which clearly agree with the data given in Figure 1.

Table 3

Diffusion Rate of K^+ and Na^+ During the First 5 Minutes in Extracts of Rat Brain in a Resting State and After Irritation (in % of min.; mean of six experiments ± standard deviations)

State	K^+	Na^+
Rest	1.58 ± 0.06	1.28 ± 0.04
Irritation	1.78 ± 0.08	1.13 ± 0.05

Although it was not quite clear to us how to interpret the results of these experiments, it seemed logical that the diffusion process had to slow down if part of the cations of the extract was combined with slowly diffusing or wholly nondiffusing anions. Since Cl^- is known to delay the diffusion of H^+, it is quite possible that fixed anion groups of proteins hold back the cations until they are unlinked. When suitably improved, the method described above will enable us to measure the cohesion between cations and proteins.

Another approach to the problem of K^+ and Na^+ combining with cell proteins involved an attempt to measure ion activity in the extracts. We conducted a few experiments with the polystyrene membranes described by Lewis and Saroff (1957). These membranes can be selectively permeable to Na^+ or K^+. The results were highly promising, even though the selectivity was not wholly satisfactory.

Better results came from experiments with glass electrodes described by Eizenman, Rudin, and Casby (1957), which Dr. Eizenman was kind enough to furnish us.

The electrode used to determine Na^+ (NaS-11-18) is 400 times more sensitive to Na^+ than to K^+. Its sensitivity to hydrogen is very slight in the physiological pH area in which we are working. The electrode used to determine K^+ (NaS-45-4/Ca 3) acts less selectively. Even so, it is eight times less sensitive to K^+ than to Na^+. Its sensitivity to H^+ is quite satisfactory. None of the electrodes reacts adequately to the NH_4^+ ions.

The electrodes were filled with a 0.1 M solution containing sodium or potassium ions. Silicon was used for insulation, and the electrode was connected to a chlorinated silver wire. Measurements were made against a standard calomel electrode (Beckman 4970-29) by means of an electrometer (Vibron Electrometer, model 33B, made by Electronic Instruments, Ltd., Richmond, Surrey, England), which we were able to use with a low input voltage (from 10 mv) and high resistance ($10^{13}\Omega$) of sources with a stability of $\pm$ 100 uv. The activity of Na^+ and K^+ was measured by the corresponding electrode in brain extracts prepared in isotonic saccharose. The concentration of ions was measured in the same extracts in a flame photometer using a standard solution. SH-groups were determined by amperometric titration.

The activity coefficient was derived from the ratio of activity to concentration. Table 4 sums up the results of 74 experiments in measuring Na^+ activity. K^+ activity was also determined in 40 experiments.

Table 4

Content of SH-Groups, Sodium, and Potassium in the Rat Brain in a Resting State and After Irritation*

Indicator	In a resting state	n	After irritation	n	p
-SH (in ug)	4.87 $\pm$ 0.58	34	7.12 $\pm$ 1.47	40	0.01
Na^+ (f	0.88 $\pm$ 0.04	34	0.74 $\pm$ 0.06	40	<0.001
(Total (in ug)	46.5 $\pm$ 4.0	34	48.0 $\pm$ 5.7	40	--
(Bound (in ug)	6.0 $\pm$ 1.65	34	13.9 $\pm$ 3.6	40	<0.001
K^+ (f	0.74 $\pm$ 0.07	20	0.89 $\pm$ 0.04	20	<0.001
(Total (in ug)	71.1 $\pm$ 6.9	34	70.0 $\pm$ 8.5	40	--
(Bound (in ug)	18.6 $\pm$ 5.6	20	8.0 $\pm$ 3.3	20	<0.001

* The mean values $\pm$ standard deviations are cited.

It is quite evident that extracts from a brain in a resting state possess high sodium activity and low potassium activity. In brain extracts obtained after electric irritation of the central ending of the sciatic nerve for 20 minutes with a frequency of 60 cps, the correlation between ion activities is reversed. The activity coefficient (f) of sodium in a resting state and of potassium after excitation tends toward a value close to that for pure NaCl and KCl in the same area of concentration (f = 0.91). These differences become very significant in connection with the determination of t. The total concentration of Na^+ and K^+ remains unchanged.

Table 4 also gives the mean values of the SH-group in the brain in resting and excited states. If they are plotted against the activity coefficients, a certain relationship will emerge. Figure 2 shows the correlation for Na^+, from which it is clear that ion activity is reduced following an increase in the number of SH-groups uncovered. The relationship for the mean values is expressed by an S-shaped curve. Figure 3 shows a similar relationship for K^+, the increased activity of K^+ ions being related to the added number of SH-groups. Figure 4 gives the mean values for both ions. It is evident that the two curves are virtually mirror reflections of each other. This result is in keeping with our thinking on exchange adsorption.

With these data in mind, we returned to the results of the experiments on diffusion described above and discovered some striking facts. It follows from Table 5 that dividing the value d determined from the slope of the straight lines in Figure 1 by the activity coefficient f of each group of experiments (the standard solution NaCl or KCl, extract from a brain found in a resting state, extract from an excited brain) yields a radio proportional to the diffusion coefficients of (D) Na^+ or K^+, respectively. This is clearly seen in the equation:

$$D = k\frac{d}{f}, \qquad (2)$$

where the constant k = 2.95, as computed from the experimental data.

Table 5

Correlation Between the Results of Measuring Diffusion and Activity

Indicator		Na^+			K^+		
		Standard Solution	In a resting state	After irritation	Standard Solution	In a resting state	After irritation
Experimental data	d	0.422	0.413	0.352	0.519	0.440	0.483
	f	0.91	0.88	0.74	0.91	0.74	0.89
	d/f	0.464	0.470	0.476	0.571	0.593	0.543
	D*	1.390	--	--	1.676	--	--
Computed data	d**	0.428	0.416	0.349	0.517	0.420	0.504
	f	0.90	0.88	0.75	0.92	0.77	0.85
	d/f	0.476	0.473	0.465	0.560	0.545	0.530
	D	1.37	1.39	1.40	1.68	1.75	1.60

*Diffusion coefficients for NaCl and KCl from the International Critical Tables (McGraw Hill, 1929, New York, 5:63).

**From the equation $D = k\frac{d}{f}$, in which k = 2.95, as computed from the experimental data.

Using this expression, we computed d from the experimental values of f and f from the experimental values of d. The new ratio thus obtained d/f was substituted in equation (2) to compute the value D. The results are obviously close to the theoretical values for NaCL or KCl in the International Critical Tables. The results of the experiments on diffusion with potassium are, generally speaking, not completely reliable; their inaccuracy may well be due to the fact that the concordance for Na^+ is better than for K^+.

The most probable explanation of these results is that some of the cellular K^+ and Na^+ are in a bound condition and these fractions change with the state--resting or excited-- of the cell. The bound fraction can be computed from the total concentration and activity coefficient given in Table 4.

The proportion of bound ions to the total number of ions is more difficult to determine in cases where the extracts include some extracellular sodium and potassium. Rat serum contains 145 u/Na^+ and 5 u/K^+ per millimeter. Since approximately 25% of the ions in the extracts are of extracellular origin, it is possible to calculate that it comes to about 15_uM of Na^+ and about 90_uM of K^+ for each gram of fresh tissue. Subsequent

calculations show that in the resting state almost 40% of the Na^+ is in bound form, whereas in the excited state almost all the sodium may be bound. However, when the cell is at rest only about 20% of the sodium is bound, but the amount of bound potassium may decrease to 10% or less after irritation.

The problem of monovalent cations bound with cell proteins has been the object of extensive research. A. S. Troshin (1956), Harris (1957), and Shanes (1958) surveyed the pertinent literature. There is no doubt that part of the K^+ and Na^+ is in a bound state and, very likely, even in several kinds of bound state. Our research has revealed a bound fraction that is electrically inactive. It is obvious that there are bound states wherein the ions retain their osmotic and electrochemical activity.

Calling the type of binding observed in our experiment adsorption explains neither the nature of the bond nor the place of the binding. However, the problem becomes somewhat clearer if we recognize that excitation causes exchange adsorption, that is, the desorption of K^+ and its replacement with Na^+. Probably the two cations are competing for the same place of adsorption. It is also possible that both are competing with H^+, if we admit the validity of Saroff's hypothesis (1957) that potassium is retained by two adjacent protein chains due to the action of the coordination links: COO-...K.../$_2$HN.

The part played by thiol groups in binding ions cannot be accurately determined. Koshtoyants (1951, 1954) stressed the unusual role of the SH-groups in nervous and muscular activity. The recent findings of Smith (1958), Reynolds, Chenoweth, and Ellman (1958), and Takahashi, Murai, and Sasaki (1958) also support the view that SH-groups have a special function. However, we tried to use our research as a basis for regarding these groups as convenient indicators of structural changes in protein molecules. These changes involve the uncovering of other side groups that may perform a more important function in ion adsorption. We have direct proof that the tyrosine OH-groups are ionized, and it is quite likely that a large number of carboxyl, amino, and imino groups are also liberated.

Working with tissue extracts had the one great advantage of enabling us to show that the ion shift during excitation is not determined by some special property of the cellular membrane, but is caused by changes in certain cytoplasmic structures. However, this method too is limited. It would be overly optimistic to expect proteins and ions to behave in extracts the way they do in living cells. This is particularly true as far as the content and state of water in living matter is concerned. The problem of "bound" water in the cell raised by Meigs (1912) has still not been solved, but some new and interesting approaches have been devised (Bozler, Calvin, and Watson, 1958: Bozler and Lavine, 1958). Szent-Györgyi (1957) mentioned the possibility of water structure losing its amorphous quality on the surface of protein molecules and forming a regular lattice characteristic of the structure of ice. According to the "fluid ice" or "iceberg" hypothesis, which has been confirmed by the recent studies of

Klotz (1958), a much greater amount of water is bound with proteins than was indicated in earlier calculations. It is also possible that the atmosphere of fluid ice surrounding the protein molecule is an important factor in preserving the true native structure and that it plays an important part in masking the side groups. We have to recognize that the structural changes induced by the excitation process may disrupt the normal structure of water and "melt" the fluid ice. It is interesting to note that the hydrated K^+ ion fits exactly into the ice lattice, whereas the Na^+ ion is too large (Buswell and Rodenbush, 1956).

We have mentioned some of the principal methods that will have to be used in solving the problem of excitation at the molecular level. There is need of further research on protein structure and its relation to the binding of ions and to water not as a simple solvent, but as a possible structural element.

Elements of the Hypothesis

Although many questions remain unanswered, I should like to set forth some considerations on the nature of excitation in the light of the observations described above.

The fact that the phenomena of excitation can be studied outside the cellular structure would seem to rule out the membrane as a sure barrier to the migration of ions. It is still debatable whether excitation can be detected outside a structure. Illumination of visual purple solutions causes chemical changes closely related to excitation (Wald, 1958) and even associated with increased conductivity (Hara, 1958). However, other types of excitation require an intact cellular structure and uninjured metabolic processes. It is also possible that excitation caused by electrical irritation is a phenomenon occurring chiefly on the surface of the cell.

To be effective, an irritant requires the existence of some cellular structure capable of absorbing its energy. If it is radiant energy, it must be absorbed by a "chromophoric group--retinene--for visible light, by aromatic amino acids for ultraviolet rays, etc. Chemical irritants are linked with receptor groups in the cytoplasm. No energy-absorbing structures have been found in connection with the action of mechanical, thermal, or electrical stimuli, but it is logical to assume that the surface elements separating the cell from the medium play an important part in the process.

The energy absorbed by specific cellular structures is obviously transmitted to the bound proteins just as the energy absorbed by retinene is transmitted to the opsinic part of the visual purple molecule. It is still unclear whether some other process precedes molecular reconstruction of the proteins, e.g., transfer of electrons (Schmitt, 1947), protons (Wirtz, 1947), initial reduction of pH (Segal, 1958), or an enzymatic process, as we are now inclined to believe.

It is generally held that the reconstruction of side groups of protein caused by changes in its molecular structure shifts the affinity of cytoplasm from K^+ to Na^+. It is unlikely that in nerves and muscles these changes take place in all the protein molecules at the same time. The data show that each impulse irritates only the surface layers of the proteins so that Na^+ is adsorbed from the extracellular fluid with the liberation of K^+. From a potassium electrode in the resting state the cell becomes a sodium electrode during excitation (Figure 5).

During the resting state the current is transferred almost entirely to the K^+ ions and the resting potential is determined by their asymmetrical distribution. In the active state the current is transmitted within the Na^+ ions and the resultant action potential is only a reflection of the nonequivalent distribution of these ions. The lower branch of the action potential comes from the outward movement of the K^+ ions which are ready to replace the Na^+ ions on the surface of the cell as soon as the resting state is restored.

Following repeated impulses the steadily increasing amount of sodium bound with proteins comes inside. This accumulation of altered proteins and bound sodium enables us to observe changes that would be undetectable if complete restoration did not follow each impulse.

Complete restoration obviously occurs only on the surface, which changes its affinity with each impulse from K^+ to Na^+ and back to K^+. The depth of the surface affected by this process, according to Aschheim's calculations (1959), is equal to the radius of the ions in the crystal. This suggests that a monoionic adsorption layer is present and that changes in the potential are caused by short-range forces of the order of the ζ-potential.

The main difference between this hypothesis and the membrane theory lies in the major role assigned to the selective adsorption of ions. It also contradicts the principal element of the permeability theory according to which all the intracellular K^+ and Na^+ ions are free. If these ions were not completely free, neither Nernst's equation nor the Goldman field equation modified by Hodgkin and Katz (1949) could be used without taking ion activity into account. The result will vary not only with the concentration of free ions, but also with the concentration of free water, which cannot be precisely computed at the present time by any method.

The hypothesis of metabolically controlled ion pumps whose function is to maintain the assymetrical distribution of sodium and potassium between the cells and in the intracellular medium is a corollary of the membrane theory. This hypothesis is apparently unnecessary for the adsorption theory, although the metabolic factors remain highly important in preserving protein structure at rest and, consequently, ionic asymmetry. Forces are constantly at work in the living cell striving to destroy this structure and disrupt the equilibrium resting state, which can be maintained only by the continuous expenditure of metabolic energy.

The "pump" hypothesis requires "carriers" to participate in the active migration of ions. According to Muralt (1958), the "carriers may exist in resting, active, and inactive forms. Substitution of carriers for the areas binding the ions (with their affinity changing from K^+, Na^+, and perhaps H^+) may provide a wholly satisfactory explanation of many changes in the phases of the action potential.

The resting state is characterized by high potential energy and low entropy, whereas energy is liberated and entropy increased in the excited state. These characteristics are particularly marked after pathological excitation, e.g., the action of bacterial toxins, antigen-antibody reactions, chemical poisons, and potent physical irritants. They may cause irreversible injury to the cell, but their principal action mechanism is identical to that in physiological excitation. Moreover, energy is absorbed with the help of the corresponding "chromophoric" group and there is molecular reconstruction of proteins that almost invariably ends in pronounced proteolysis (Ungar, 1956; Ungar and D mgaard, 1954, 1955; Ungar and Hayashi, 1958) and an ion shift. This last embrances not only K^+ and Na^+, but also important organic substances like histamine, 5-oxytryptamine, and various products of protein decomposition. Histamine is obviously associated with cellular proteins like potassium and is released when the proteins assume a freer configuration.

We are aware that many parts of our hypothesis may turn out to be idle reflections, but we hope that some of them will be subjected to direct experimental verification.

Conclusion

The significance of changes in the configuration of protein molecules has now been substantiated by research in many branches of biology. Our work has corroborated Nasonov's view that excitation causes changes in cellular proteins that resemble denaturization. It is quite probable that structural changes in these molecules control the affinity of the side chains for potassium or sodium. Our recent investigations show that in a resting state cellular proteins chiefly adsorb K^+, but Na^+ when in an active state. Adsorption of these ions is qualitatively connected with the number of free SH-groups, which can be regarded as indicators of the adsorption properties of the protein molecule.

An attempt was made to explain the mechanism of excitation in the light of these observations. The energy of the irritant absorbed by certain cellular structures is transferred to the proteins, causing the highly organized native molecular structure to weaken. This results in replacement of the adsorbed K^+ with Na^+ and thus initiates the electrical phenomena of excitation. These phenomena are superficial in nerves and muscles since each impulse affects only the surface layer of the proteins and ions. Although this hypothesis differs radically from the widely held membrane theory, many divergencies are semantic in nature, which can be understood from a careful recheck of certain fundamental conceptions, e.g., that of membrane permeability. The adsorption hypothesis, it seems to us, is much more economical in its assumptions and more readily verifiable by direct experimentation.

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CHANGES IN THE SIZE OF MESOTHELIAL AND MAST CELL NUCLEI FOLLOWING THE ACTION OF NOVOCAIN

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L. G. Vol'fenzon
Laboratory of Cellular Adaptations,
Institute of Cytology, Academy of Sciences USSR
Leningrad

A study of the effect of local anesthetics on different tissues in vitro (Vol'fenzon, 1954) showed that novocain in concentrations used for anesthesia was capable of inducing reversible changes not only in nerve cells, but also in cells of other kinds of tissues (suppression of granulation in rat kidney and pancreas cells and rabbit connective tissue, halting of ciliary movement of rat trachea cells, muscle belly contracture of the grass frog abdomen).

The action of novocain on fibroblasts and histiocytes of rabbit areolar tissue resulted in the suppression of granulation and contraction of cell bodies and their nuclei. These changes turned out to be reversible since the cells became normal after the novocain was washed out in Ringer's solution. The reversible contraction of connective tissue cells due to novocain was later studied quantitatively in some detail (Aleksandrov and Vol'fenzon, 1956). It appears that the degree of cell contraction varies with the concentration of the irritant. Moreover, subsequent calculations showed that the nuclei as well as the cytoplasm are compressed, the degree depending on the novocain concentration. Fibroblast and histiocyte contraction closely resembles muscular contracture and is evidently an expression of local persistent excitation.

The effect of novocain on the cells of non-nerve tissues was extensively studied by P. P. Rumyantsev (1959). He showed that the action of novocain in various concentrations (0.0125 to 2%) on frog corneal epithelium and skin, mouse liver, and some other tissues in the cytoplasm near the Golgi apparatus is followed by the formation of visible, intravitally colorless inclusions of novocain resembling the granules of vital stains. The author conjectures that the formation of these inclusions is a defense reaction, which is important in that it removes the injurious agents from the cell.

Thus, our data and Rumyantsev's investigations show that novocain is not a purely neurotropic agent, as hitherto believed by most authors (Nikolayev, 1943; Vishnevskiy and Vishnevskiy, 1948), and that like other substances it is capable of inducing major changes in tissues.

Since novocain is widely employed in visceral surgery, we thought it would be interesting to study the effect of this anesthetic on tissues of the serous membranes, particularly in view of references in the literature to the high sensitivity of these tissues to a great variety of irritants.

We selected as the object of research the mesothelial cells of rat mesentery and the mast cells found in large numbers in the connective layer of the rat mesentery.

Due to reversible contraction of the protoplasm and nuclei of connective tissue cells following the action of novocain, we decided to study its effect on mesenteric tissue with respect to changes in the size of mesothelial and mast cell nuclei. We confined ourselves solely to measuring the nuclei because the boundaries of mesothelial cells are not always clearly demarcated when the preparations are processed by the usual histological methods.

Material and Methods

The experiments were performed in vitro at room temperature (17° to 21°). The prepared mesentery was cut into individual segments, some of which (the control) were placed for an hour in Earl's solution, which is now extensively used for cultivating tissues outside the organism, while the rest were kept first for 30 minutes in Earl's solution, then for 30 minutes more in novocain solutions of varying concentration prepared in Earl's fluid (from one to three concentrations were tested in each experiment). The control and experimental segments were then fixed in osmic acid fumes and stained with Yasvoyn's hematoxylin. To determine whether the changes in the cell and nuclei caused by the novocain were reversible, we returned one of the segments that had been kept in novocain for 30 minutes to pure Earl's solution for 1½ to 2 hours and then fixed it.

We evaluated the changes in the tissue caused by the irritant from contraction of the nuclear area. We used a drawing device to project the nuclei onto paper of standard thickness. Relative changes in area were determined by weighing an identical amount of experimental and control copied and excised nuclei. Thus, we have given all the sizes in conventional units (milligrams of paper weight).

The degree of contraction was determined from the difference (expressed as a percentage of the control set at 100%) between the arithmetic means of 100 measured nuclei in the control portions of the mesentery and 100 measured nuclei in the portions of the nuclei subjected to the action of novocain. The degree of reversibility, consequently, was the difference between the arithmetic means of 100 nuclei from the control portions of the mesentery and 100 nuclei from the portions of the mesentery transferred from the novocain solution to Ringer's solution. Each experiment was statistically processed and the mean errors of the control nuclei and those groups of nuclei subjected to the action of novocain were determined. The mean contraction of the nuclei (as a percentage of the control) from all the experiments and the standard deviation were then computed for each concentration.

A total of 50 series of experiments were performed and 8000 nuclei measured.

Mesothelial Nuclei

Mesothelial cell nuclei were measured after segments of mesentery were kept for 30 minutes in 0.05, 0.1, 0.5, and 1% novocain solutions in Earl's fluid. Table 1 shows that even after the action of 0.05% novocain there was a rather significant contraction in some experiments. However, in other experiments this concentration scarcely had any effect. Thus, the mean size of the nucleus for all the experiments was reduced 10% all told.

Changes of the same order were caused by 0.1% novocain. In only three out of six experiments did this concentration result in nuclear contraction, the compression for all the experiments averaging 7%.

In all the experiments 0.25, 0.5, and 1% novocain solutions caused statistically reliable nuclear contractions. The mean percent c concentration was 22, 24, and 40 after 0.25, and 0.5, and 1% solutions, respectively (Table 2 cites the effect of the 0.5% solution on the mesothelial nuclei).

Table 1

Changes in the Size of Mesothelial Cell Nuclei After the Action of a 0.05% Solution of Novocain

Arithmetic mean of nuclear size (from 100 nuclei) in standard units		$M_{dif} \pm m_{dif}$	Contraction of nuclei (as % of the control)
Control	Experiment		
40.1	37.5	2.6 ± 0.54	6.0 ± 1.3
40.4	39.3	1.1 ± 0.62	2.7 ± 1.5
52.9	39.5	13.4 ± 1.0	25.0 ± 1.9
48.5	35.9	12.6 ± 0.87	26.0 ± 1.8
40.7	40.2	0.5 ± 0.65	0.12 ± 0.15
41.0	40.4	0.6 ± 0.80	0.15 ± 0.20
36.4	32.4	4.0 ± 0.94	11.0 ± 2.6
Arithmetic mean of all seven experiments and standard deviation			10.0 ± 1.1

Table 2

Changes in the Size of Mesothelial Cell Nuclei After the Action of a 0.5% Solution of Novocain

Arithmetic mean of nuclear size (from 100 nuclei) in standard units		$M_{dif} \pm m_{dif}$	Contraction of nuclei (as % of the control)
Control	Experiment		
46.6	38.4	8.2 ± 1.05	16 ± 2.2
43.2	27.6	15.6 ± 1.3	36 ± 3.0
49.0	36.8	12.2 ± 1.3	25 ± 2.7
52.9	35.7	17.2 ± 0.9	33 ± 1.7
48.5	28.1	20.4 ± 0.84	43 ± 1.7
36.4	28.4	8.0 ± 0.8	23 ± 2.2
Arithmetic mean of all six experiments and standard deviation....			24 ± 2.4

Figure 1 shows distribution curves of the sizes of the nuclei in one of the experiments in which portions of mesentery were placed in 0.25, 0.5, and 1% novocain solutions, respectively. It is evident from the figure that all three concentrations cause nuclear contraction (the distribution curves shift toward the smaller values).

The mean contractions of mesothelial cell nuclei are brought together in percentages of the control for all the experiments in composite Table 3 (left side) to compare the effect of the different concentrations of novocain. The table shows that the degree of contraction increases with the concentration (from 7 to 10% in 0.5 to 0.1% solutions of novocain to 40% in 1% novocain).

Mast Cell Nuclei

After measuring mast cell nuclei in a few experiments, we found that they contracted reversibly after the action of the same concentrations of novocain like the mesothelial nuclei (i.e., from 0.1 to 1%; Table 3, right side). A 0.1% solution caused slight contraction of the nuclei (7%). Contraction increased to 33% after a 0.25% solution. It unexpectedly turned out to be somewhat lower (14%) in 0.5% novocain. However, in 1% novocain it increased again (48%). Thus, the degree of mast cell compression likewise grows with increases in concentration.

Table 3

Contraction of Mast Cell Nuclei as a Percentage of the Control After the Action of 0.05, 0.1, 0.25, 0.5, and 1% Novocain Solutions

Concentration of novocain (as %)	Mesothelial nuclei		Mast cell nuclei	
	total number of nuclei measured in all the experiments	arithmetic mean of the value of nuclear contraction (as%)	total number of nuclei measured in all the experiments	arithmetic mean of the value of nuclear contraction (as %)
0.05	1400	10 ± 1.1	--	--
0.1	1600	6.9 ± 1.1	200	7 ± 3.3
0.25	1800	21.8 ± 2.5	600	33 ± 3.4
0.5	1200	24 ± 2.4	400	14 ± 1.7
1	400	40 ± 1.8	200	48 ± 2.3

Figure 1b shows the distribution curves of the sizes of mast cell nuclei after the action of 0.25, 0.5, and 1% novocain solutions. The curves indicate that all three concentrations caused nuclear contraction (the curves shifted toward the smaller values). A comparison of this figure with Figure 1a shows that the nature of the curves and the degree of contraction of mast cell nuclei and mesothelial nuclei are quite similar.

The contraction of mesothelial nuclei and mast cell nuclei caused by novocain proved to be reversible. After the segments of mesentery were transferred from 0.25 and 0.5% novocain solutions back into Earl's fluid, the sizes of the nuclei again increased, sometimes regaining their original values.

Table 4 presents data on reversibility of the action of 0.25 and 0.5% novocain solutions on mesothelial nuclei, while Figure 2 (a and b) shows the contraction of mesothelial and mast cell nuclei after they were subjected to the action of 0.5% novocain (the distribution curves of the nuclei shift toward the smaller values) and return to their former size after the novocain was washed out in Earl's fluid (the distribution curves again approach the controls).

Table 4

Reversibility of the Action of 0.25 and 0.5% Novocain Solutions on Mesothelial Nuclei

Course of the Experiment	Time of action (in min)	Arithmetic mean of the value of the nuclei (from 100 nuclei in conventional units)	Contraction of nuclei (as % of the control)
Earl's solution	30	43.2 ± 0.75	0
0.25% novocain in Earl's solution	30	27.2 ± 0.65	37
Reversibility. Earl's solution	90	33.7 ± 0.65	22
Earl's solution	30	46.6 ± 0.86	0
0.25% novocain in Earl's solution	30	38.4 ± 0.61	18
Reversibility. Earl's solution	90	47.2 ± 1.1	0
Earl's solution	30	43.2 ± 1.1	0
0.5% novocain in Earl's solution	30	27.6 ± 0.5	36
Reversibility. Earl's solution	90	33.2 ± 0.9	23

Conclusion

Our data and the results of earlier studies (Vol'fenzon, 1954; Aleksandrov and Vol'fenzon, 1956) indicate that in the cells of certain tissues (rabbit areolar tissue, rat serous membranes) substantial changes caused by novocain may lead to reversible contraction of the protoplasm and nuclei. However, data in the literature and our researches show that many other agents besides novocain may induce reversible contraction of protoplasm and nuclei in various tissues.

Thus, reversible contraction of rat areolar tissue in vitro also took place after the action of $CaCl_2$, KCl, ethyl alcohol, and some other irritants (Aleksandrov and Vol'fenzon, 1956). Several investigators likewise noted the contraction of fibroblasts in vivo under a great variety of experimental influences (Möllendorf and Möllendorf, 1926; Yasvoin, 1948; Yeliseyev, 1948). Mesothelial cells have been described as rounded, compressed detached (in vivo) as a result both of physiological (mechanical pressure of internal organs) and of artifical (various chemical agents, trauma) irritants (Shchelkunov, 1936; Khlopin, 1937; Baron, 1940, 1948). The same cells were contracted in films obtained from the mesentery and omentum of guinea pigs under the influence of adrenalin and some other pharmacological agents (Niessing, 1938). There are also data on the decreased size of mast cells after inflammation (Maksimov, 1904) and after the action of cortisone (Asboe-Hansen, 1952).

There are, moreover, many reports on the size of nuclei in certain tissues when subjected to various actions. The nuclei shrink in the parietal and chief cells of the mucuous membrane in the stomach of rats after vagotomy and injection of histamine; in liver cells due to daily fluctuations in glycogen content (Caspersson and Holmgran, 1934); in the cells of the cerebrospinal ganglia of frogs and mice in vitro when exposed to the action of ether or ethyl alcohol; in cells of cultures of renal glomeruli in vitro following the action of ether (Krantz, 1947), etc.

These data indicate that the substantial changes caused by a variety of irritants in the cells of certain tissues may induce reversible contraction of the protoplasm and nuclei. This phenomenon is therefore an additional sign of lesion whose presence is particularly significant when the usual methods of determining the functional state of cells cannot be employed.

The method of studying granulation after intravital staining of tissues with basic vital dyes is, of course, widely employed to determine the functional state of cells. However, it is not always suitable because in certain cells the protoplasm cannot combine with the granules of basic stains. According to V. Ya. Aleksandrov (1949), the mast cells that we studied in connection with this report are an example of cells from which granules of neutral red cannot be obtained.

We showed earlier (Vol'fenzon, 1954) that local anesthetics (when used in normal concentrations--0.25 to 2%) cause reversible lesion in renal and pancreatic cells and in rabbit connective tissue cells. Comparing these facts with those obtained in the present investigation, we see that the tissues of serious membranes are highly sensitive to novocain since the nuclei of mesothelial and mast cells contract slightly (7 to 10%) following the action of even weaker concentrations (0.05, 0.1%). This fact will undoubtedly have to be taken into account when the effects of anesthetization of the abdominal cavity are analyzed.

Findings

1. Novocain in concentrations similar to those employed in medicine causes the nuclei of mesothelial cells to contract.

2. The degree of nuclear contraction increases with the concentration of the novocain; 0.05 and 0.1% solutions cause only slight contraction (averaging 10% of the control); contraction increases to 24% after the action of 0.25 and 0.5% solutions and amounts to 40% in 1% solution.

3. Measurements of mast cell nuclei showed that these nuclei contract under the influence of the same concentrations of novocain as mesothelial nuclei.

4. The contraction of mesothelial and mast cell nuclei following the action of novocain is reversible since the nuclei again enlarge, sometimes reaching the size of the control, after the pieces were transferred from the novocain solution to Ringer's solution.

5. The results of this study are in agreement with the data obtained when we investigated the effect of novocain on the epithelium, kidneys, and pancreas of rats and on the areolar tissue of rabbits. They prove once again that novocain is not a purely neurotropic agent and that like other irritants it is capable of inducing nonspecific changes in various tissues.

6. These data also show that the reversible contraction of the nuclei observed in certain cells is a highly sensitive indicator of lesion. Changes therein make it possible to take into account not only the nature, but also the degree of action of an irritant on cells.

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THE EFFECT OF VITAMIN B_{12} ON LIVER CELLS IN VARIOUS FORMS OF EXPERIMENTAL DYSTROPHY

[Pages 649-652]

R. A. Petrov
Department of Pharmacology,
Blagoveshchensk Medical Institute

Vitamin B_{12} has been widely employed in recent years to treat patients suffering from pernicious anemia. There are data on the use of this vitamin for liver diseases as well. L. A. Cherkes (1953) refers to the experiments of Hull and Drill in which fatty degeneration of the rat liver induced by improper diet was prevented from developing by preparations containing vitamin B_{12}. Cherkes reports that large doses of the vitamin may mitigate changes in the liver even in the case of toxic dystrophy caused by carbon tetrachloride. The regenerative ability of liver cells may also play a part in the restoration of liver tissue to normalcy after nutritional or toxic dystrophy as well as in the processes of fat and lipoid metabolism. This problem too has been discussed in the literature, but Ferrari and Brondoni, who investigated it (1954), believe that vitamin B_{12} has no effect on the regeneration of liver cells.

The objective of our research was to determine the effect of vitamin B_{12} on liver cells in dystrophic processes brought about by various means.

The work involved the use of intravital staining of specimens obtained by puncture. This enabled us to study the origin and elimination of liver degeneration in the same animal. Intravital staining of cells makes it possible to determine their functional state (degree of damage) since, according to D. N. Nasonov and V. Ya. Aleksandrov (1940), the character of the intravital stain is an indicator of the cellular state.

Experiments were performed on 90 white rats. Before starting we examined liver punctures of all the animals. In the first series of experiments liver degeneration was induced in animals taken away from their mothers by putting them on a protein-free diet of starch, sucrose, salt, vegetable oil, and water. The second series of experiments was performed on mature rats in which degeneration was induced by adding 25% fat and 1% cholesterol to the food, which was generally unlimited. Analysis of the punctures and administration of vitamin B_{12} began after the animals were kept on the diet for 30 days. In the third series of experiments involving both adult and baby rats toxic liver dystrophy was induced by feeding the animals orally with a 50% solution of carbon tetrachloride (0.3 ml per 100/g of body weight) every 24 hours for three days. Meanwhile, the rats stopped eating their regular food and became dirty and sluggish. The doses were about the same as those recommended by K. A. Meshcherskaya (1954) to produce toxic fatty degeneration of the liver. Her research (1952, 1954) showed that the number of pathologically changed cells increased 50% in a hepatocytogram on the fourth day after these doses of carbon tetrachloride were administered.

Vital staining of the liver specimens obtained intravitally with a fine needle was done with neutral red. A 1% solution of the stain in absolute alcohol was first applied to a defatted slide. After the slide dried completely, the specimen was placed on it, covered with a cover glass, and set in a moist chamber at a temperature of about 30°. t least 200 cells were counted in each tap. A detailed description of the method and a comparison of it with the technique of counting hepatocytograms on fixed preparations were given in an earlier paper (Petrov, 1957).

The results obtained in the first series of experiments on 40 baby rats are shown in Tables 1 and 2.

Table 1

Number of Paranecrotic Liver Cells (as %) in Baby Rats Kept on a Protein-Free Diet (the rats were returned to a normal diet after the 14th day)

Substance administered	Days of experiment				
	0	7	14	21	28
Distilled water (0.3 ml per 100 g of body weight). Control.....	0	39 ± 0.3	63 ± 5	37 ± 1	4.5 ± 0
Vitamin B_{12} (5 ug per 100/g of body weight, daily).....	0	44 ± 2	66 ± 4	17 ± 0.5	0

Table 2

Number of Paranecrotic Liver Cells (as %) in Baby Rats After 14 Days of Protein Starvation

Substance administered	Days after halting protein starvation			
	0	7	14	21
Distilled water (0.3 ml per 100 g of body weight, daily). Control..	75 ± 2	39 ± 3	9 ± 0.4	4 ± 0.5
Vitamin B_{12} (5 ug per 100/g of body weight, daily)....	78 ± 3	10 ± 0.5	0	0

It is evident from Tables 1 and 2 that the number of paranecrotic liver cells in baby rats kept on a protein-free diet increased, reaching 63 to 75% by the 14th day. The administration of vitamin B_{12} during protein starvation (5ug/per/100/g of body weight, daily) did not prevent these changes. Hepatocytograms taken on the same day revealed cells with drops of fat and indications of granular dystrophy, amounting to 60% of all the liver cells. When the rats were returned to a normal diet two weeks after the experiment began (Table 2), a significant difference showed up between the experimental and control animals. In the animals that received Vitamin B_{12} the original state of the liver cells was restored more quickly.

The second series of experiments was performed on 20 adult rats. Vitamin B_{12} was administered for 21 days in doses of 5ug/per 100/g of body weight daily. The results are presented in Table 3.

Table 3

Number of Paranecrotic Liver Cells (as %) in Rats Receiving 1% Cholesterol and 25% Fat in Their Food

Substance administered	Days of the experiment			
	30	37	44	51
Distilled water (0.3 ml per 100/g of body weight, 21 days). Control.....	29 ± 3	40 ± 2	23 ± 3	46 ± 2
Vitamin B_{12} (5 ug/per 100/g of body weight, 21 days).....	29 ± 5	31 ± 4	35 ± 5	52 ± 6
M ± m diff.	--	9 ± 2.8	12 ± 2.4	6 ± 5.7

Besides liver speciments obtained by puncture, we also examined in this series of experiments blood taken from the tail, using the method of intravital staining. We showed earlier (1957) that paranecrosis develops in leukocytes from drops of tail blood in fatty degeneration of the liver induced by starvation and tannic acid. This happens at the same time that paranecrotic cells appear in the liver. Unlike other forms of liver affections, with cholesterol dystrophy the number of paranecrotic leukocytes did not exceed 10% of all the cells in any of the experiments, i.e., it was the same as in healthy animals.

The third series of experiments was performed on 30 rats. Toxic liver dystrophy was induced by carbon tetrachloride. Vitamin B_{12} was administered to one group of animals after dystrophic changes were detected in the liver; it was given to another group together with carbon tetrachloride. Besides liver specimens obtained by puncture, we examined drops of tail blood. The results of this series of experiments are presented in table 4.

Table 4

Number of Paranecrotic Liver Cells (as %) in Rats with Liver Dystrophy Induced by Carbon Tetrachloride

Substance administered	Days of the experiment			
	0	4	7	14
Carbon tetrachloride (0.3 ml per 100/g of body weight, 3 days). Control....	0	88 ± 1	53 ± 4	9 ± 0.5
Carbon tetrachloride (0.3 ml per 100/g of body weight, 3 days) + vitamin B_{12} (5ug per 100/g of body weight, 13 days).....	0	78 ± 4	20 ± 2	0
Carbon tetrachloride (0.3 ml per 100/g of body weight, 3 days) + vitamin B_{12} (5ug per 100/g of body weight, 10 days).....	0	88 ± 4	16 ± 1	0

Note. The number of paranecrotic leukocytes reaches 60% on the fourth day of the experiment.

The table shows that the administration of vitamin B_{12} with carbon tetrachloride does not prevent the development of toxic dystrophy of the liver. After administration of the carbon tetrachloride is discontinued, vitamin B_{12} hastens the return of the liver cells to normalcy.

It is evident from the results of the first and third series of experiments that vitamin B_{12} hastens the return of liver cells to normalcy after administration of the injurious agent is discontinued. (In the second series of experiments (with cholesterol dystrophy of the liver) we were unable to test the effect of vitamin B_{12} for this purpose because the control animals were used elsewhere for biochemical and pathohistological experiments.) This result in all probability is nonspecific in character since dystrophic processes caused by protein starvation and intoxication differ in origin and are not the consequence of vitamin deficiency.

Findings

1. Vitamin B_{12} does not prevent changes in liver cells resulting from a protein-free diet, but it does hasten the return to normalcy when the ordinary diet is restored.

2. Vitamin B_{12} administered simultaneously with cholesterol does not change the degree of dystrophic changes in liver cells.

3. Vitamin B_{12} hastens the return of liver cells to normalcy after toxic dystrophy caused by carbon tetrachloride.

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THE STATE OF INORGANIC PHOSPHORUS IN PROTOPLASM

[Pages 653-660]

L. N. Pisareva
Laboratory of Cellular Physiology,
Institute of Cytology, Academy of Sciences USSR,
Leningrad

Chemical analysis indicates that the concentration of so-called "inorganic" phosphorus in protoplasm is much higher than in the blood plasma surrounding the cells (cf. Fenn 1936; Ling, 1952; Troshin, 1956a; others). Experiments with radioactive phosphorus show the intensive exchange of various phosphorus fractions of the cell (including fractions of inorganic phosphorus) with phosphorus of the labelled orthophosphate of the surroundings. However, the specific radioactivity of mineral phosphorus in the cell is invariably lower than its specific radioactivity of many organic phosphorus cellular compounds (Hahn and Hevesy, 1942; Mullins, 1942; Mueller and Hastings, 1951).

This phenomenon has still not been satisfactorily explained. Some believe that the cell membrane is not permeable to the anion of orthophosphoric acid. Others hold that phosphorus on the cellular surface is first esterified after which it passes through the cell membrane in the form of organic phosphorus compounds (Kamen and Spiegelman, 1948; Causey and Harris, 1951; Mitchell, 1953, 1954, 1957; Goodman and Rothstein, 1957). Still others maintain that the anion of orthophosphoric acid freely penetrates into the cell where it is incorporated into the organic compounds (Furchgott and Shorr, 1943).

Some investigators have concluded that the concentration of free mineral phosphorus (anion of orthophosphoric acid) in the cell is much lower than that indicated by chemical analysis. They assume that the basic mass of phosphorus determined chemically as "inorganic" exists in a nondissociated state and that it may well be an artifact caused by the decomposition of labile organic phosphorus compounds during the extraction of phosphate from the tissue (Kamen and Spiegelman, 1948; Elliott and Hevesy, 1950; Chambers and White, 1954).

It follows from the above that the mechanism of phosphorus exchange between the cell and surroundings cannot be explained unless one knows the states in which intracellular phosphorus may occur. It is particularly necessary to determine with maximum accuracy the amount of its inorganic (free) fraction.

We studied in connection with this report the distribution of phosphorus between the frog sartorius muscle and physiological solution containing labelled orthophosphate. At the same time we determined chemically the inorganic and total phosphorus of the muscle. Finally, we tried to calculate the actual amount of the free inorganic phosphorus fraction in the muscle.

Method

Experiments were performed on the sartorius muscles of the grass frog from October to May. Inorganic phosphorus was determined by the method of Fisk and Subarrou after freezing in liquid oxygen. Total phosphorus was determined by the same method after preliminary mineralization. The rate of phosphorus exchange of the muscle with inorganic phosphorus of the surroundings was studied with the help of P^{32} in the form of $Na_2HP^{32}O_4$.

Prepared muscles were placed for one hour in a nonradioactive solution, which was made up just before the experiment by mixing equal quantities of two solutions as follows: solution 1 -- NaCl = 111.1 mM, KCl = 2.5 mM, $CaCl_2$ = 1.8 mM; solution 2 -- Na_2HPO_4 = 2.5 mM, NaH_2PO_4 = 0.5 mM (Keynes, 1954). This solution contained 9 mg% phosphorus. The muscles were transferred to the same solution where part of the Na_2HPO_4 was replaced with $Na_2HP^{32}O_4$. The muscles were taken out of the solution at various intervals and the activity of their phosphorus determined. Specimens to calculate the activity were prepared in the way described by Troshin (1956b, 1957).

The concentration of phosphorus in the muscles displaced by labelled phosphorus of the surrounding solution was computed per 100 g of wet tissue weight and per 100 g of intracellular water. The wet tissue weight was computed from the formula

$$C_m = C_8 \times Q,$$

where C_m = concentration of phosphorus displaced in the muscles, C_8 -- concentration of phosphorus in the outer radioactive solution, Q -- distribution coefficient $\left(Q = \frac{\text{counts/min. per g of wet muscle weight}}{\text{counts/min. per ml of outer solution}}\right)$.

The intracellular water was computed from the formula

$$C_c = \frac{C_8 (Q-0.1)}{0.7},$$

where C_c is the concentration of displaced phosphorus computed for the water of the muscular fiber; the other symbols are the same as in the formula above. In the computations 0.7 g out of each g of wet tissue weight was allowed for the water of the muscular fibers and 0.1 g for the water of the intercellular spaces (Troshin, 1956a).

The results of the experiments were statistically processed; the mean arithmetic and mean square errors were determined.

Results

1. Changes in the content of total and inorganic phosphorus in the muscles while in the saline solution. Two series of experiments were set up to determine the total and inorganic phosphorus in the muscles immediately after preparation and after different periods of immersion in physiological solution. In the first series the muscles were kept in saline solution at a temperature of 18°; in the second series they were kept in the same solution cooled to 2° C.

We found in the frog sartorius muscle freshly removed from the organism 267 ± 15 mg% total phosphorus (8 experiments), 38.9 ± 1.9 mg% inorganic phosphorus (18 experiments). The phosphorus content was then determined every one or two hours, the last determination being made 8 hours after the muscle was immersed in the physiological solution. Table 1 shows the values obtained. No regular changes were observed in the total phosphorus content of the muscle during the experiment. The inorganic phosphorus content of the muscles shifted perceptibly during the first 90 minutes they were immersed in the physiological solution (to 54.3 ± 2.6 mg% at 18° and to 30.0 ± 2.4 mg% at 2°), evidently the result of a change in the functional state of the muscles due to preparation and removal from the organism. The inorganic phosphorus later approached the original content (46.1 ± 4.2 mg% at 18° and 41.4 ± 2.7 mg% at 2°)

Table 1

Changes in the Content of Total and Inorganic Phosphorus in Muscles While Immersed in Physiological Solution

Period of incubation	Temperature							
	18°				2°			
	number of experiments	total phosphorus (in mg%)	number of experiments	inorganic phosphorus (in mg%)	number of experiments	total phosphorus (in mg%)	number of experiments	inorganic phosphorus (in mg%
0	8	267 ± 15	18	38.9 ± 1.9	8	267 ± 15	18	38.9 ± 1.9
90	2	302 ± 1	14	54.3 ± 2.6	3	271 ± 36	8	30.0 ± 2.4
120	3	229 ± 8	10	46.1 ± 4.2	3	296 ± 17		
180		--	10	44.8 ± 4.5		--	6	41.4 ± 2.7
240	3	264 ± 20		--	3	266 ± 30		--
300		--	10	47.2 ± 2.9		--	6	43.8 ± 5.3
360	3	254 ± 11		--		--		--
420		--	10	48.8 ± 5.8		--		--
480	3	290 ± 27		--	3	249 ± 65		--

2. Distribution of labelled phosphorus between frog muscles and surrounding saline solution. We set up four series of experiments to study the displacement of P in the muscles by radioactive phosphorus of the surrounding solution under normal conditions (temperature 18°) and with suppression of enzymatic processes (addition of sodium azide to the medium, cooling to 2 and 0°). In each series the activity of the phosphorus was measured 15, 30, 60, 120, 180, 300, and 420 minutes after the muscles were placed in the radioactive saline solution. The results of the experiments are shown in Table 2. Each value in the table is the mean of 8 to 12 determinations.

The main series of experiments was performed at 18°, repeated twice (at the beginning and at the end of the work); the results of both groups of determinations coincided completely. Figure 1, curve I shows that radioactive phosphorus penetrated rapidly into the muscle during the first 15 minutes (1.27 ± 0.07 mg% of muscle phosphorus displaced) after which the process slowed down. In general, after seven hours only 7.6 mg% muscle phosphorus was displaced by labelled phosphorus of the surrounding solution. This totaled about 16% of the amount of inorganic phosphorus determined at this time by chemical means (48.8 mg% and 2.6% of the amount of total muscle phosphorus--290 mg%).

These data are insufficient to indicate the distribution of labelled phosphorus between inorganic and organic fractions of the muscle However, one may assume that if phosphorus penetrates into the cell in the form of orthophosphate, suppression of enzymatic reactions should not greatly affect the rate of uptake of labelled phosphorus by the muscles since much less labelled phosphorus penetrates into the muscles during experiments at 18° than is necessary to displace the "mineral" fraction. On the other hand, if orthophosphate does not penetrate into the muscle fibers and phosphorus penetration into the muscle fibers is due to esterification on the cell surface, suppression of enzymatic reactions should lead to a sharp drop in the rate of labelled phosphorus uptake by the muscles. With sufficient inhibition the maximum amount of displaced muscle phosphorus should not exceed the amount of this anion found in the intercellular spaces.

However, experiments that were run to solve the problem failed to support either hypothesis. Suppression of enzymatic reactions by adding sodium azide to the physiological solution in which the muscles were immersed and lowering of the temperature to 2 and 0° substantially reduced the rate of labelled phosphorus uptake by the muscles, as is evident from curves II, III, and IV in Figure 1. During the first 30 minutes labelled phosphorus entered the muscles fairly rapidly, then slowed down, and sometimes after 3 hours ceased altogether. It is also clear from the shape of curves II, III, and IV in Figure 1 that the amount of displaced muscle phosphorus in all three cases invariably attains a value greater than what the intercellular spaces could include. Experiments at 2 and 0° yielded identical results.

Table 2

Amount of Muscle Phosphorus Displaced by Labelled Phosphorus of the Surroundings Under Various Experimental Conditions

Experimental conditions	Period of incubation (in min.)			
	15	30	60	120
$NaN_3 = 3.3 \times 10^{-3}$ M, 18°	1.27 ± 0.07	1.40 ± 0.08	1.89 ± 0.13	2.61 ± 0.17
$NaN_3 = 3.3 \times 10^{-3}$ M, 18°	1.32 ± 0.09	1.54 ± 0.10	2.05 ± 0.12	2.77 ± 0.23
$NaN_3 = 3.3 \times 10^{-3}$ M, 2°	0.73 ± 0.09	0.83 ± 0.13	1.16 ± 0.06	1.51 ± 0.[illegible]
$NaN_3 = 3.3 \times 10^{-3}$ M, 0°	0.90 ± 0.06	1.06 ± 0.07	1.26 ± 0.15	1.40 ± 0.[illegible]

	180	300	420
$NaN_3 = 3.3 \times 10^{-3}$ M, 18°	3.81 ± 0.21	5.65 ± 0.42	7.58 ± 0.26
$NaN_3 = 3.3 \times 10^{-3}$ M, 18°	3.12 ± 0.25	4.17 ± 0.15	3.48 ± 0.28
$NaN_3 = 3.3 \times 10^{-3}$ M, 2°	1.63 ± 0.06	2.27 ± 0.11	2.42 ± 0.14
$NaN_3 = 3.3 \times 10^{-3}$ M, 0°	1.54 ± 0.09	2.08 ± 0.28	2.39 ± 0.14

Results of the experiments involving suppression of enzymatic processe support the assumption that (1) mineral phosphorus may penetrate into muscle fibers in the form of an anion of orthophosphoric acid (if our conditions ensure adequate inhibition of the enzymatic processes) and (2) the concentration of free mineral phosphorus of muscle fibers found in diffusion equilibrium with orthophosphate of the surrounding medium is much lower than the concentration of muscle "mineral" phosphate determined chemically.

In the following series of experiments we tried to determine the true value of the free mineral phosphorus fraction in muscle fibers.

3. Relationship between the concentration of muscle phosphorus displaced at low temperatures and its concentration in the surrounding saline solution. The concentration of a substance dissolved in a cell under the conditions of diffusion equilibrium is directly dependent on its concentration in the surrounding medium. This rule applies to the distribution both of nonelectrolytes and of organic and mineral ions (Troshin, 1956a).

The curves in Figure 1 show that when enzymatic reactions are suppressed, the growth in concentration of labelled phosphorus in muscles almost comes to a complete halt in 5 hours. It is probable that a diffusion equilibrium has set in between the labelled phosphorus of the surrounding medium and the displaced phosphorus of the muscle. Under these conditions it is possible to determine the value of the phosphorus fraction dissolved in the protoplasm of the muscle fiber. This requires the construction of a graph showing the relationship between concentration of labelled muscle phosphorus and orthophosphate concentration in the surrounding medium, as was done by A. S. Troshin (1956a, 1957) for other substances.

To study this relationship, we ran a series of experiments in which isolated frog sartorius muscles were kept for 420 minutes in saline solutions chilled to 2° and containing 2.25 mg% phosphorus ($\frac{1}{4}$ the normal content), 9 mg% phosphorus (normal), and 18 mg% phosphorus (double the normal). We compensated each time for the change in amount of phosphorus in the solution with an equimolecular amount of sodium chloride. Muscles held in these solutions did not differ from the controls in irritability, water content, or external appearance. Theconcentration of displaced muscle phosphorus was computed per 100 g of wet tissue weight and per 100 g of intracellular water. The results of this series of experiments are given in Table 3 and Figure 2. The straight line ab in Figure 2 represents the relationship between concentration of displaced muscle phosphorus (C_c) and concentration of orthophosphate in the equilibrium surrounding medium and corresponds to the formula

$$C_c = C_s K + A_{oa},$$

where A_{oa} and K are constants. Here the total amount of displaced muscle phosphorus (C_c) is obviously made up of dissolved (C) and bound (A) (by adsorption or chemically) fractions. The constant A_{oa}, as is evident from the graph, is numerically equal to the segment on the ordinate oa. In our case A_{oa} is equal to 0.8 mg%. This is the maximum amount of bound muscle phosphorus (A) that can be displaced under our conditions by labelled phosphorus of the surrounding medium.

It is a fair assumption that the straight line oc parallel to straight line ab (Figure 2) reflects the relationship between concentration of phosphorus dissolved in muscle fibers (C) and concentration of orthophosphate in the surrounding medium (C_s) and agrees with Henry's law $C_c = C_s \times K$, where k is the constant of proportionality and equal to the tangent of the angle of slope of straight line oc. It shows the degree to which orthophosphate solubility in protoplasm differs from its solubility in the water of the surrounding solution. In this instance K = 0.17, i.e., regardless of the orthophosphate concentration in the surrounding medium, its concentration in the water of the muscle fiber under the conditions of diffusion equilibrium is 83% less than in the surrounding solution.

Very low values of the constant K were obtained by Troshin, who studied the distribution of different nonelectrolytes, macromolecular organic electrolytes, and mineral ions between frog muscles and saline solution surrounding them (Troshin, 1956a). On the basis of similar data other authors (Nasonov and Aleksandrov, 1943; Troshin, 1956a; Oparin, 1957; others) conjectured that water in the protoplasm is in a special state, possessing low solvent ability.

The area of the intercellular spaces cannot be determined easily, but it is safe to assume that it is no more than 10% for the frog sartorius muscle (Troshin, 1956a). Assuming further that the concentration of salts in the intercellular fluid is the same as in the surrounding medium, we come up with 0.9 mg% inorganic phosphorus for the intercellular spaces since the phosphorus content of our solution was 9 mg%.

Water averages 80% of the weight of sartorius muscle; after deducting 10% for water in the intercellular spaces we are left with 70% protoplasmic water. We computed the coefficient of phosphorus solubility in the protoplasm as 0.17. Hence, 1.1 mg% phosphorus (9 x 0, 17 x 70%) may be dissolved in the water of the protoplasm. This means that the muscle contains 2 mg% free inorganic phosphorus (0.9 mg%) dissolved in the water of the intercellular spaces and 1.1 mg% in protoplasmic water. Even if a later, more precise determination should reveal that the area of the intercellular spaces is greater or less than 10%, the resultant corrections in our computations would be trivial. (Hahn and Hevesy (1942) found 1.5 mg% free phosphorus in rabbit erythrocytes.)

Discussion

In recent years some investigators (Troshin, 1956a; Briner, Shirley and Shaw, 1958; others) advanced the view that muscle phosphorus is distributed entirely among three fractions: (a) phosphorus dissolved in intercellular fluid, (b) free intracellular phosphorus (dissolved in protoplasm), and (c) bound phosphorus in the cell (adsorbed and incorporated in inorganic phosphorus compounds). The data presented in this paper tend to support this hypothesis.

About 40 mg% inorganic phosphorus was found in frog sartorius muscle by chemical methods (Fenn, 1936; Panteleyeva, 1953; this paper). However, our calculations showed that only 2 mg% phosphorus is to be found in a free state and, consequently, the basic mass of phosphorus determined chemically as "inorganic" is in a free state in muscle fibers. Part of the so-called "inorganic" phosphorus in muscles may be represented by the phosphorus adsorbed on the proteins--colloids of the protoplasm. Data on the ability of proteins in a solution to bind orthophosphate by adsorption favor this assumption (Kometiani, 1948; Cannan and Levy, 1950; Haurowitz, 1950; Ling, 1942). Nor can we exclude the possibility mentioned above that part of this chemical fraction of phosphorus belongs to some labile organic phosphorus compounds that decompose when the phosphorus is extracted from the cells (Kamen and Spiegelman, 1948; Elliott and Hevesy, 1950, 1950; Chambers and White, 1954).

Since out of the total cellular phosphorus (267 mg%) only an insignificant portion (2 mg%) is found in a free, dissolved state, it is obvious that the rate of penetration of labelled phosphorus into the cell and its exchange with intracellular phosphorus will necessarily depend almost entirely on the rate of exchange of those organic compounds of which it forms part. It is natural, therefore, under the conditions in which the action of enzymatic systems responsible for the synthesis and restoration of organic phosphorus compounds is suppressed (lowering of temperature, addition of enzymatic toxins) that there should be inhibition of phosphorus exchange between the muscle and the surrounding medium, as we observed in our experiments.

Summary

An investigation was made of the distribution of labelled phosphorus between isolated frog sartorius muscle and a surrounding saline solution at a temperature of 18° and under conditions involving the suppression of enzymatic processes (action of sodium azide, lowering of the temperature of the solution to 2 and 0°). At the same time inorganic and total phosphorus in the muscle were chemically determined. It was discovered that only a small part (2 mg%) of the phosphorus chemically determined as "inorganic" (40 mg%) was in a free state in the cell.

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THE POLARIZATION COEFFICIENT OF MUSCLES SUBJECTED TO THE ACTION OF FORMALIN

/Pages 661-664

Ye. V. Burlakova
Department of Biophysics, Moscow University

Electric polarization of tissue after a current is passed through it is regarded as a characteristic of its native condition. Changes in polarizability following lesion, injury, and death of protoplasm may serve as a sensitive indicator of changes in its physicochemical structure, as demonstrated by B. N. Tarusov (1938).

Following the action of substances normally used as fixatives by histologists, tissue polarizability, as in ordinary necrosis, is sharply reduced. Formalin is well known as a special fixative. For example, D. N. Nasonov and his co-workers (Nasonov and Aleksandrov, 1944; Nasonov and Speranskaya, 1948) showed that the injury potential characteristic of living nerve and muscle may be found in nerve and muscle treated with a 20% formalin solution. Other reports (Spiegel-Adolf, Henny, and Ashkenaz, 1944; Kozlov, 1958) contain indications that formaldehyde used as a fixing fluid scarcely changes the roentgenogram. We know that nerves treated with formalin are the best material for transplantation of nervous tissue (Anokhin, 1953). Finally, the specific nature of formalin action on tissue is revealed by the fact that muscles fixed with formalin retain their polarization capacity (Tarusov, 1941).

In a discussion of the results described in some of the papers cited (Tarusov, 1941: Nasonov and Aleksandrov, 1944) it was conjectured that formalin does not destroy the structure of protoplasm that determines the electric polarization of tissue. However, the processes taking place in living tissues treated with formalin have not yet been fully elucidated. Nor is it known whether the degree and nature of polarization of tissues treated with formalin vary with the histological structure and functional characteristics of these tissues. The purpose of our investigation was to determine how the polarization properties of muscles differing in structure and possessing various functional properties might change when fixed with formalin.

We used for experimental objects the crab (Carcinus maenas) flexor muscle of the extremities, the obturator muscles of the common mussel (Mytilus edulis) and oyster (Ostrea taurica). We analyzed the relationship between electric resistance and frequency as an index of polarizability. The so-called polarization coefficient suggested by B. N. Tarusov (1938) was the measure of polarizability. This coefficient shows how much less tissue resistance measured at a frequency of 1 meg. is than the resistance measured at a frequency of 10 kc. $K = \frac{R_{10^4}}{R_{10^6}}$.

Muscle resistance was measured with a special apparatus constructed on the principle of bridge circuits. The apparatus was manufactured in the workshop of the biophysics department of Moscow University and consisted of a measuring bridge and a sinusoidal voltage generator to power it. The frequency band of the generator ranged from 25 c. to 1 meg. The generator supply came from a stabilizer. The measuring bridge included two fixed high-stability resistors with three and six commutator [17] and standard boxes: a resistance box MSRB-48 and two capacitor boxes ME-3 and ME-6. The tangential electrodes of the measuring chamber were attached to one of the arms of the bridge. The apparatus contained two separate bridges mounted on a single panel to measure resistance at high and low frequencies. These were connected to the measuring circuit by a toggle switch. A resonance circuit installed at the outlet of the bridge was fastened to the panel. The generator was connected to the bridge through a decoupling transformer, which was also mounted on the general panel. An EO-7 cathode oscillograph was used as the zero indicator of the measuring bridge. Measurement error did not exceed 1%. Muscle resistance was measured at frequencies of 0.2, 2, 10, 20, 10 c., and 1 meg. to determine the frequency relationship.

Muscles were placed at the tangential platinum electrodes in a moist chamber made of plexiglass. Measurements of the muscles immersed in formalin (4 and 8% solutions) were made every day during the first month of experimentation and periodically during the second month. We performed a total of six series of experiments. In the first three series we studied the relationship between magnitude of resistance and frequency at frequencies ranging from 0.2 to 20 kc. (100 experiments on crab, 80 on mussel, and 80 on oyster muscles). (These experiments were performed at the Sevastopol' Biological Station of the Academy of Sciences USSR. Measurements were made with a bridge supplied by a 3G-10 transformer. Resistance box R-14.) In the second three series the investigation was conducted at the higher frequencies--from 0.2 kc to 1 meg.--and the polarization coefficient K computed. There were 170 experiments all told (100 on crab, 40 on mussel, and 30 on oyster muscles).

A comparison of the results of all the experiments shows that the muscles retained their polarization capacity throughout the experiment, i.e., more than 2 months, following prolonged immersion in 4 and 8% formalin. The polarization coefficient usually diminished by the end of the second month. The following table gives the changes in polarization coefficient of crab, mussel, and oyster muscles treated with 4% formalin solution.

Changes in the Polarization Coefficient of Muscles Following Prolonged Immersion in 4% Formalin

No. of series and object	No. of experiment	Polarization coefficient				
		original	after immersion in formalin			
			after 2 days	after 5 days	after 1 month	after 2 months
4. crab muscle	1	2.7	2.6	2.85	2.85	2.25
	2	2.6	2.4	2.7	2.9	2.1
	3	2.75	2.6	2.8	2.8	2.1
	4	2.85	2.85	2.9	2.8	2.3
	5	2.55	2.5	2.7	3.1	2.5
	6	2.9	2.9	2.9	2.95	2.5
	7	2.8	2.7	2.85	3.05	3.01
	8	2.6	2.5	2.85	2.85	2.45
	9	2.65	2.7	2.8	2.65	2.05
	10	2.7	2.5	3.1	3.4	3.0
	Mean	2.72	2.62	2.83	3.02	2.4
5. Mussel muscle	1	2.4	2.4	2.4	2.4	2.0
	2	2.3	2.2	2.3	2.4	1.9
	3	2.5	2.4	2.5	2.5	2.5
	4	2.3	2.3	2.3	2.1	1.8
	5	2.2	2.3	2.3	2.0	1.6
	6	2.1	2.0	2.0	2.2	1.7
	7	2.6	2.5	2.5	2.1	1.6
	8	2.5	2.7	2.5	2.2	1.5
	9	2.5	2.5	2.5	2.5	2.2
	10	2.6	2.8	2.6	2.7	2.2
	Mean	2.4	2.4	2.4	2.3	1.9
6. Oyster muscle	1	2.3	2.3	2.4	1.9	1.5
	2	2.3	2.3	2.4	2.5	1.8
	3	2.2	2.2	2.3	2.0	1.7
	4	2.3	2.2	2.3	2.3	1.8
	5	2.2	2.2	2.4	2.4	1.9
	6	2.4	2.3	2.4	2.0	1.7
	7	2.3	2.3	2.4	2.0	1.7
	8	2.4	2.4	2.5	2.5	2.5
	9	2.4	2.4	2.5	2.4	2.0
	10	2.3	2.4	2.4	2.5	2.1
	Mean	2.3	2.3	2.4	2.2	1.8

It is evident from the table that crab muscle had the largest original polarization coefficient and oyster muscle the least. During immersion of crab muscle in 4 and 8% formalin solutions the polarization coefficient decreased slightly at first (a decrease was noted in 70% of the cases), but then increased. An increase was not noted in all the experiments and usually after two months it was again followed by a decrease. The number of experiments in which a decrease in the coefficient occurred during the formalinization process was substantially less for mussel and oyster muscles. Thus, a decrease in polarization coefficient of mussel and oyster muscles on the third day of immersion in formalin was observed in 40% and 20% of the experiments, respectively. However, these changes were insignificant.

The table presents the results of 30 experiments. The results were the same in the other experiments. The difference lies only in the absolute values of the magnitudes of resistance and the polarization coefficient, which apparently depend to a large degree on the physiologic state of the object and the conditions under which it is kept.

The high value of the polarization coefficient of crab, mussel, and oyster muscles after prolonged immersion in formalin suggests that the polarization structure of these muscles is preserved after they are treated with formalin. This conclusion is also supported by the results of determining the entire curve of resistance changes at frequencies ranging from 0.2 kc. to 1 meg.

The figure shows the relationship to frequency of crab and mussel muscles before formalinization and on the sixth day of their immersion in 4% formalin solution. It is evident from the curve that the decreased resistance following increased frequency in formalinized muscles is similar to that in the controls. The difference between the two is simply that the absolute value of resistance is somewhat less in the formalinized muscles. Comparable results were obtained in the other series.

We may conclude from a comparison of the data that formalin fixes the structure of muscle tissue that determines electric polarization. Moreover, this polarization structure is preserved after formalinization of muscles differing in origin, histological structure, and functional properties. The difference determined experimentally in changes of polarization coefficient values between crab muscle on the one hand and mussel and oyster muscles on the other is a valid basis for assuming some difference in the physicochemical properties of the structure that determines the polarization of these two groups of muscles. This difference is apparently due to the different concentration and condition of the ions in muscular tissue. This fact has been reported in the literature (Aladzhalova, 1950, 1956).

In conclusion, I should like to thank the directors of the Sevastopol' Biological Station of the Academy of Sciences USSR for the facilities and materials they placed at my disposal for this research.

Findings

1. An investigation was made of the polarization properties of certain marine animals--crab (Carcinus maenas), mussel (Mytilus edulis), and oyster (Ostrea taurica)--after treatment with 4 and 8% formalin solutions.

2. It was established that the relationship between magnitude of resistance of crab, mussel, and oyster muscles and frequency is retained after prolonged immersion in formalin.

3. The resultant data justify the conclusion that formalin is capable of fixing the physicochemical structure that provides the polarization properties of muscles with different functional characteristics and a different histological formation.

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TRANSMISSION OF EXCITATION IN THE GIANT AXON OF THE SQUID /Ommatostrephes Sloonei-pacificus (Steenstrup)/

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A. A. Lev, N. N. Nikol'skiy, D. L. Rozental' and Ye. A. Shapiro
Laboratory of Cellular Physiology, Institute of Cytology of the Academy of Sciences USSR, Leningrad

We showed in an earlier article (Lev, Nikol'skiy, Rozental', and Shapiro, 1959) that the relationship between magnitude of local electric response and intensity of irritation in the giant axon of the squid is gradual in nature and graphically expressed by an S-shaped curve. We also showed that after deterioration of the functional state following survival of the fiber in a moist chamber the S-shaped curves tend to slope more toward the axis of abscissas. The relationship thus established between strength of irritation and magnitude of reaction is in complete agreement with the principles of the gradual theory of excitation developed by D. N. Nasonov (1959).

The pattern of conduction of nerve impulses along elementary parts of the fiber emerges from the gradual relations between the magnitudes of irritation and response characteristic of the fiber. After weak subliminal irritation the impulse travels along the fiber, decreases, and at some distance from the site of irritation disappears (decremental conduction of excitation). After supraliminal irritation the impulse spreads and grows until it attains a constant magnitude. Before this happens the response will decrease if the tissue is irritated by stimuli that elicit a response exceeding the traveling spike in magnitude. Thus, incremental or decremental conduction of a stimulus may be observed near the site of irritation if the stimulus is supraliminal. It will be transmitted further with unchanged amplitude only at some distance from the site of stimulation once it has attained a controlled magnitude. This is the nature of conduction as assumed by the gradual theory of excitation for a normally functioning conducting fiber.

Deterioration of the functional state causes changes in the quantitative relationship between intensity of irritation and magnitude of electric response. At first there is a decrease in amplitude of the controlled impulse and an increase in threshold of the local response. This is followed by disappearance of the capacity for nondecremental conduction and the tissue responds to any irritation solely by decrementally spreading excitation.

A number of facts support the principles of conduction suggested by the gradual theory. Almost all the investigators of subliminal local response have observed decremental conduction (Hodgkin, 1938; Schmitt and Schmitt, 1940; Rosenbluth and Luco, 1950; Stämpfli, 1952). The spatial decrement of excitation caused by strong supraliminal stimuli was studied by M. S. Averbakh and D. N. Nasonov (1950) on the frog sciatic nerve. Similar data are cited in the article of Schmitt and Schmitt (1940). This paper contains evidence of spatial increment of excitation.

The nature of impulse conduction in the anesthetized nerve has long been a matter of dispute. D. N. Nasonov and D. L. Rozental' (1952) recorded action currents on the oscillograph, observing the shift from nondecremental conduction to decremental conduction in the case of frog sciatic nerve totally anesthetized by the fumes of alcohol, ether, chloroform, or ammonia. We shall analyze the reasons for the contradictions in all the early data on the condution of impulses in the anesthetized nerve.

It will be noted that experimental evidence in support of the principles of conduction postulated by the gradual theory is not of equal value. Decremental conduction of subliminal excitation has been investigated fairly fully while spatial increment has scarcely been studied. Decrement following supraliminal excitation and the transition from nondecremental conduction to decremental conduction have been investigated only on the whole nerve trunk. We therefore deemed it necessary in this paper to verify certain principles governing the conduction of impulses in the giant axon of the squid /Ommatostrephes Sloonei-pacificus (Steenstrup)/.

The basic procedures in working with the single giant axon of the squid are described in a separate paper (Lev, Nikol'skiy, Rozental', Svinkin, and Shapiro, 1959). An isolated fiber was placed in a moist chamber on a grid of platinum electrodes spaced 1.5 to 3 mm. apart. Irritation was effected with square pulses of 100 microsecond duration and 25 c. frequency. The action potentials were led off with two pairs of electrodes helped by a two-channel amplifier with balanced input. A two-gun cathode-ray oscillograph with photo attachment was used for recording purposes. Each exposure lasted 0.3 to 0.5 sec., i.e., 7 to 12 run-downs of the beam were fixed for each frame.

The experiments were performed at room temperature (19 to 21°).

The nature of Conduction in Fibers Functioning Normally

The potentials were led off after irritation of the nerve with stimuli of varying intensity, beginning with weak or subliminal ones. The oscillograms in Figure 1B (frames 13, 14, 15) show that after irritation of subliminal force (0.154, 0.184, 0.212 v.) there was a response only under the near electrode of the first pair, the magnitude growing with intensification of the stimulus. Thus, after subliminal irritation the electric response arising under the cathode spread for several millimeters and became attenuated somewhere between the first and second electrodes of the near pair. This was the picture observed in most of the experiments. However, it was sometimes possible to catch the moment when the excitation reached the second electrode of the first pair, becoming attenuated somewhere between the near and distant pairs of electrodes. In this case the screen of the oscillograph showed a biphasic response recorded only by the near electrodes (Figure 1B, frames 16 and 17). The presence of a biphasic response reveals the shift of a limited area of negativity along the fiber, i.e., it suggests that decremental conduction of excitation is effected by the same mechanism as the conduction of a regulated wave. Hodgkin (1938) was inclined to adopt this view on the grounds that a subliminal local response spreads further than an electrotonic potential.

Subsequent intensification of the stimulus causes the wave of excitation to reach the second pair of electrodes (Figure 1B, frame 18). (Intensification for the potentials led off from the near and distant electrodes was different. Hence their amplitudes cannot be compared.)

Figure 1C shows oscillograms of a similar experiment. Here, when the irritation was intensified, there was also an increased response in the area of the near electrode and then a shift of the local response to the spreading spike. Moreover, fluctuations in the magnitude of response were noted under the near electrodes, whereas the response in the area of the distant electrodes had a constant magnitude. With irritation of threshold intensity a subliminal reaction and fluctuating spike (Figure 2A, frames 30 and 31) are frequently recorded in one frame. The oscillations in magnitude of spike can be explained by fluctuations in excitability, as we pointed out in an earlier report (Lev, Nikol'skiy, Rozental', and Shapiro, 1959). It should be stressed that this picture is observed under the near electrode of the first pair, less commonly under the second electrode, and least of all or never at the far electrodes (second pair). We see, therefore, that the constant magnitude of the spike is determined only at some distance from the cathode.

Let us analyze in more detail the oscillograms of action potentials following threshold irritation. Figure 2A, frames 33 and 34, and Figure 2B show oscillograms which indicate that when the magnitude of response found in the area of the distant electrodes and under the second electrode of the near pair is constant, a response of different magnitude is recorded under the first electrode of this pair. We stress the fact that we are dealing here not with a subliminal response that becomes attenuated at some distance from the cathode, but with an action potential spreading over the entire length of the fiber. In this case the presence of a different response under the first electrode with constant amplitude of the second phase can be understood only as incremental momentum of the spike. (As mentioned above, the fluctuation in excitability is the most probable cause of the oscillation in magnitude of the spike. Thus, insignificant gradations in the relative force of the stimulus caused by fluctuations in excitability lead to changes in the magnitude of the response, which is revealed with exceptional clarity after irritation of threshold force.)

It follows from the gradual theory that the shorter the S-shaped magnitude of response-intensity of irritation curve, the narrower is the area of supraliminal force of irritation at which a response may develop above the threshold, but below the maximum, i.e., the area of incremental momentum. We have shown that an S-shaped curve is steep for fibers in a good functional state (Lev, Nikol'skiy, Rozental', and Shapiro, 1959). The incremental momentum of the spike may be observed in this case with very small shifts in the force of irritation from the threshold value due to fluctuation in excitability. An example of this spatial increment of excitation is shown in the oscillograms of Figure 2A (frames 33 and 34) and Figure 2B (frame 7). With increased force of irritation a constant magnitude of the traveling impulse is led off under all four electrodes (Figure 2A, frame 32).

We constructed a graph to illustrate the phenomena of incremental and decremental conduction of responses. The magnitude of the response under the first and second electrodes of the first pair was computed as a percent of the maximum possible response under the particular electrode. These values were laid off along the axis of ordinates, while the distances in millimeters from the cathode were laid off along the axis of absciasas. Figure 3 shows the graphs drawn on the basis of two experiments. Since the graphs have only two experimental points each, they were connected by a straight line to show the direction of change in magnitude of the response. However, we must assume on the basis of theoretical considerations that changes in the magnitude of spreading reaction along the length of the conductor are dependent not on a square, but on another, probably exponential relationship.

Decremental conduction of a subliminal response was observed in 47 cases, incremental momentum of the spike in 30 cases.

Changes in the Nature of Conduction Following Survival of the Fiber in a Moist Chamber.

An isolated axon may retain excitability when kept in a moist chamber 6-7 hours. Excitability and magnitude of response often change only toward the end of this period, remaining5-6 hours virtually at the same level. In some experiments we noted a sudden disappearance of conduction all along the conductor, but in most cases conduction disappeared in the area under the distant electrodes while a response was being recorded at the near pair. This could be interpreted as decremental conduction of excitation.

To prove that we were not dealing here with partial necrosis of individual segments of the fiber, we set up three experiments in which the lead electrodes were left at the same place while the position of the irritating electrodes was changed. If there were a genuine decrement of conduction, the magnitude of response would have to decrease in proportion to the distance of the irritating electrodes from the lead electrodes. Figure 4 shows the oscillograms of one of the experiments and the resultant graph of changes in magnitude of response in relation to the distance between electrodes. The distance in millimeters between the cathode and lead electrodes is laid off along the axis of abscissas while the magnitude of response is laid off along the axis of ordinates. It is evident from the figure that the closer the irritating electrodes are to the lead electrodes, the greater is the magnitude of response, both under the first (curve I) and under the second (curve II) electrodes. When the irritating electrodes were drawn back 9.9 mm., the response was recorded only under the first electrode, i.e., conduction became extinguished in the area between the first and second electrodes.

This relationship of magnitude of reaction to distance between irritating and lead electrodes indicates that decremental conduction of excitation follows deterioration of the functional state of the fiber.

Conclusion

This report has presented data on spatial conduction of impulses in a single nerve fiber of the squid. The decremental conduction of excitation observed after irritation of subliminal force fully accords with data in the literature.

The increment of conduction that emerges from the gradual theory and which contradicts the views of those who support the law of "all or nothing" has still not been specifically investigated. Thus, it seems particularly worthwhile to us to establish the fact of incremental growth of excitation in connection with irritation of subliminal force.

Data on decremental conduction of excitation in a single fiber surviving in a moist chamber confirm the findings of D. N. Nasonov and D. L. Rozental' (1952) on the anesthetized frog sciatic nerve. The decrement in impulses after deterioration of the functional state in a single nerve fiber excludes the possibility of explaining the results for the entire nerve trunk by statistical summation of the activity of individual fibers.

It follows, therefore, that the principles of conduction postulated by Nasonov's gradual theory of excitation reflect the actual way in which nerve impulses are transmitted.

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SEASONAL CHANGES IN THE RESISTANCE OF PLANT CELLS TO THE ACTION OF VARIOUS AGENTS

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V. Ya Aleksandrov, M. I. Lyutova and N. L. Fel'dman
Botanical Institute imeni V. L. Komarov of the Academy of Sciences USSR and Institute of Cytology of the Academy of Sciences USSR, Leningrad

In earlier papers (Aleksandrov, 1956; Aleksandrov and Fel'dman, 1958; Lyutova, 1958; Fel'dman, 1959) we reported the following fact after investigating the leaves of several plants.

1. Heating causes reversible alteration of cells in which there is a reactive increase in the resistance of the protoplasm--thermal hardening. Cell resistance increases not only to the injurious effect of heat, but also to the action of acetic acid, ethyl alcohol, and high hydrostatic pressure. The nonspecificity of heightened resistance after thermal hardening is not absolute, e.g., it does not increase resistance of the cell to ammonia. The condition caused by hardening is reversible and resistance returns in 5 to 7 days almost to its original level.

2. Increased resistance to heat and alcohol was noted in epidermal and parenchymal cells adjacent to the surface of a cut, i.e., in cells subjected to wound irritation.

3. Certain winter grasses have heat-resistant cells. We observed the same phenomenon in the Khibiny Mountains in the summer after a night frost. All this led us to start systematic observations of seasonal changes in plant cell resistance to certain injurious influences. The purpose was to discover whether cold hardening of plants under natural conditions results in increased cell resistance similar to the resistance that we induced experimentally by heat hardening. The answer to this question is essential if we are to understand the cytophysiological mechanisms responsible for the fall heightening of cold resistance in perennial plants. Although the problem is of primary importance both for the theory of cell adaptation and for practical agriculture, it is still very far from being solved. The extensive literature on the subject is full of contradictions (cf. Maksimov, 1913-1952; Tumanov, 1940; Levitt, 1945, 1956a, b).

Objects and Methods Used in the Investigation

Dactylis glomerata L., Elymus arenarius L., E. angustus Trin, and Hepatica nobilis Schreb were the principal plants studied. The were growing in an open place on the grounds of the Botanical Institute imeni V. L. Komarov of the Academy of Sciences USSR in Leningrad. In three of these areas we investigated the epidermal cells of the adaxial surface of the leaf sheaths; in H. nobilis we investigated the epidermal cells of the abaxial surface of the leaf blades. The action of injurious agents and subsequent microscopy were performed in all cases not on sections but on pieces cut from the leaf.

The plants' resistance to high temperature, high hydrostatic pressure and ethyl alcohol was tested at different times in the year. To determine resistance to heat and high hydrostatic pressure, bits of leaves were first squeezed into a syringe (Aleksandrov, 1954) with tap water and then exposed to 5 minutes of the factors mentioned above. The pieces were heated in tap water in a thermostat kept at a preset temperature of around 0.1°C. (Aleksandrov, 1955). The pieces were always transferred to the thermostat from room temperature. High hydrostatic pressure was created by a school hydraulic press in the chamber of a thick-walled steel cylinder (Golovina, 1955). A test tube with water containing the objects being investigated was placed in the water.

After 5 minutes of heating or action of high hydrostatic pressure the presence or absence of protoplasm movement was determined from the shifting of spherosomes. The resistance of the object was shown by the value of the maximum temperature or pressure after which it was still possible to observe forward movement of the spherosomes in at least one cell of the piece of leaf after 10 minutes of microscopy. To determine this value, successive tests were made at temperature intervals of 0.4° and, in experiments with pressure, at intervals of 100 atm. The resistance of cells to ethyl alcohol was determined by infiltrating the pieces with a solution of alcohol and tap water and leaving them there. The pieces were periodically examined under the microscope. Resistance to alcohol was shown by the amount of time the protoplasm continued to move in an alcohol solution of a given concentration.

Experimental evaluation of the cold-resistant parts of a plant is always tentative because the effect of chilling depends on a variety of circumstances. The results have shown that the method described below was completely adequate for a comparative evaluation of cold resistance of the same objects in different seasons. The method was as follows. Pieces of leaves were placed in silicone oil (VIZh 94a) and impregnated with it. Asahina (1956) pointed out that it is better than paraffin for use in studying the effect of low temperatures because, among other things, they scarcely increase its viscosity. Our experiments showed that silicone oil is a fairly inert medium for plant cells, which survive under a thin film of it just as well as in tap water changed daily. For example, in some experiments protoplasmic movement in the epidermal cells of tradescantia leaves impregnated with and kept in silicone oil continues for 3 months and more. Moreover, since it has a higher refractive index than water (1.44 instead of 1.33), many objects can be examined under the microscope more easily when they are in silicone oil than in water.

For cooling we used a microrefrigerator made by the Institute of Semiconductors of the Academy of Sciences USSR (Figure 1). The device is a cylinder 11.5 cm. high and 8 cm. wide. Inside is a hollow aluminum cylinder 8 cm. high and 4 cm. wide. An insulating layer of foam plastic is found between the side of the inner cylinder and the metal case of the refrigerator. A semiconductor thermobattery mounted on the base of the device does the cooling. The device is supplied by direct current and uses about 30 a with a voltage of 0.7 v. A selenium rectifier is used to feed

the microrefrigerator. The hot junction of the thermobattery is cooled by running water. The temperature inside the chamber of the refrigerator is regulated by changing the current in the power supply circuit with the help of a laboratory autotransformer. The temperature in the refrigerator chamber may be lowered to -35°. With regulated water current and stabilization of the power supply the temperature can be maintained at any prescribed level. About half an hour is required to lower room temperature to the minimum. The transition from one temperature regime to another in an operating refrigerator is much quicker, taking only 10 to 15 minutes. (It is our pleasant duty to thank Ye. A. Kolenko, Director of the Laboratory of Thermoelectric Cooling, Institute of Semiconductors of the Academy of Sciences USSR, for placing this microrefrigerator at our disposal.) The great advantages in working with a semiconductor refrigerator as compared with cryohydric mixtures to create low temperatures are obvious. The device was covered on top with a cork lid containing an opening in the center for the thermometer.

To perform the experiments, the chamber of the refrigerator was filled with silicone oil and a low temperature created. Pieces of leaf were pricked with very fine double-edged needles. After the cover of the device was raised, the needles were attached to the inner surfaces of the cover in such a way that the pieces came in direct contact with the mercury bulb of the thermometer. The cover was quickly put into its place. The pieces were kept in the chamber at a given temperature for 5 minutes, after which they were taken out and promptly transferred to the silicone oil at room temperature. We then determined the presence or absence of movement of the protoplasm. (I. M. Kislyuk was very helpful to us in determining cold resistance, for which we extend to him our sincerest thanks.)

Some time ago N. A. Maksimov (1913) pointed out that the difference in temperatures that kill different cells in the same tissue may amount to several degrees. We encountered the same variability in our own experiments and therefore evaluated cold resistance as follows. Three pieces at a time were taken from one leaf for cooling. We determined the minimum temperature after 5 minutes of which cells in all three pieces were found to have protoplasm still moving. As an additional criterion we found the maximum temperature after which the protoplasm of cells in all three pieces was motionless. In determing cold resistance the temperature to which the objects were cooled in successive tests differed by about one degree. Seasonal curves of cold resistance derived from all four objects mentioned above with the help of the first criterion (minimum temperature at which the protoplasm in all three pieces continued to move) are virtually paralleled, differing by 2 to 4°. Since we are interested solely in the dynamics of changes in cold resistance at various times of the year, we shall present only the material that was obtained with the first criterion.

The pieces placed in water or alcohol solution were examined microscopically with an aqueous immersion, apochromatic lens x70, ap. 1.23. In working with silicone oil we used an oil immersion x90 ap. 1.25.

To determined the resistance of Dactylis glomerata and Elymus arenarius cells, the material was taken from the same thickly overgrown turf. The young leaves of these grasses sprout early in the spring and remain throughout the summer. By fall some of them wither while others remain green under the snow. The number of old leaves in which the distal parts generally die off varies with the nature of the winter. In the winter and spring we always used the sheathing portionsof the leaves as long as part of the blades remained alive. The overwintered leaves of H. nobilis frequently show large necrotic patches. A continuous rapid flow of protoplasm takes place in the summer in the epidermal cells of al our plants. In the winter the flow does not cease, it merely slows down according to the drop in temperature. In the winter we did not bring the pieces of leaves into the laboratory, but placed them in already chilled silicone oil and studied them out-of-doors under the microscope after taking steps to maintain the original temperature of the objects. Viewing them under the microscope at -3°, we were sure that the cytoplasm of the cells looked the same as usual. We saw no signs of separation of the cytoplasm. The spherosomes traveled very slowly along the walls or strands intersecting the vacuolar area. The cytoplasm of H. nobilis and E. arenarius moved unusually slowly. When the pieces were taken into the laboratory, the protoplasm in all the plants at once began to move rapidly in response to the rise in temperature. Thus, "forced dormancy" directly caused by low temperature takes place in the epidermal cells of all four species of plants judging by the movement of cytoplasm in the winter. In contrast, other species of plants in the cells of which the cytoplasm moves rapidly in the summer exhibit in the winter a cessation of movement that is unaffected by a rise in temperature. Cytoplasmic movement in some of these plants resumes only after a more or less prolonged stay in the laboratory (foliar epidermal cells of Bergenia crossifolia L. Fritsch, Asarum europaeum L., Achillea millefolium L., Hesperis matronolis L., and others). One must therefore conclude from the cytoplasmic movement that the depth of winter dormancy of epidermal cells differs markedly from species to species. We are now making a special study of this problem.

Figures 2 to 12 show the data on cell resistance, each point representing the average of at least three experiments performed on different leaves.

Seasonal Changes in the Resistance of Epidermal Cells in the Sheathing Part of Grass Leaves.

Dactylis glomerata. Heat resistance of the cells was determined periodically between summer 1955 and spring 1958. The results are shown in Figure 2. They reveal that from mid-May to mid-September heat resistance is minimal and ranges from 44.0 to 44.7°. During the latter part of September (1956), but a little later in some years (1955), heat resistance begins to increase, attaining its maximum at the end of November: the

movement of protoplasm ceases only when the temperature is over 46°. High heat resistance is maintained until the end of March. It begins to drop rapidly in April, reaching the summer level by mid-May. In early spring the sheath not only of last year's leaves but also of the new leaves possesses high heat resistance. However, it decreases more rapidly in the cells of young leaves so that early in May there is a substantial difference between the heat resistance of cells in the new (the corresponding dots are encircled) and the old leaves. As the leaves develop, heat resistance again increases, and by the latter part of May it reaches the normal summer level in young leaves.

The data in Figure 2 relate to heat resistance determined from the maximum temperature at which movement can still be observed after 5 minutes of heating. The variation of the protoplasm-temperature curves characterizes more fully the cell heat resistance (Aleksandrov, 1956). Figure 3 shows similar curves for the summer (I) and winter (II) Dactylis. The curve is shifted to the right in accordance with the greater heat resistance of winter Dactylis. However, the shape of the curve and its slope, which is determined by the temperature coefficient of heat injury, remain unchanged.

Seasonal changes in resistance to high hydrostatic pressure coincide with changes in heat resistance (Figure 4). The minimum resistance to this factor also occurs during the active life of the plant. Resistance to alcohol was observed in unsystematic fashion for a year. The action of 8% alcohol was studied in December 1957 and June 1958 and of 12% alcohol in June 1956 and December 1957. It is evident from the table that protoplasmic movement in winter plants in 12% and 8% alcohol continues 27.3 and 7.6 times longer than in summer plants.

Elymus arenarius and E. angustus. In these grasses cell resistance to high temperature, intense hydrostatic pressure, and alcohol changes during the season just as it does in D. glomerata, as shown in Figure 5 (A and B) and in Table 1.

Table 1

Resistance to Ethyl Alcohol by Epidermal Cells of the Leaf Sheath

Species	Number of Experiments		Concentration of alcohol (in %)	Period movement continues (in Min.)		Ratio of periods: winter / summer
	summer	winter		summer	winter	
Dactylis glomerata	27	24	12	66	1800	27.3
The same	9	9	8	2113	16020	7.6
Elymus arenarius	15	24	12	38	5640	148.4

For our main investigation of these three grasses we used the epidermis of the leaf sheath and not the blade because the intravital structure of epidermal cells is more clearly visible in the sheath than in the blade. Individual experiments showed, however, that epidermal cells in the blades became more resistant by winter. For example, from June to August the heat resistance of these cells in E. arenarius was 43.2%, while resistance to high hydrostatic pressure was 1100 atmospheres (average of 2 experiments). Other values were obtained at the end of October -- 44.5% and 1300 atm., respectively (average of 9 experiments).

Hence we may conclude that at the beginning of fall the foliar epiderral cells of D. glomerata, E. arenarius, and E. angustus exhibit sharply increased resistance to high temperature, high hydrostatic pressure, and ethyl alcohol. This heightened resistance remains throughout the winter; it diminishes in the spring and by mid-May returns to the summer level.

The Relationship Between Heightened Grass Cell Resistance in the Fall and Temperature

The question naturally arises as to the cause of the general increase in cell resistance in the fall. Is it the direct result of the drop in temperature? Shortening of the day? Developmental phase of the plant? We ran the following experiments in an effort to answer the question.

Early in May 1957 we planted D. glomerata in several dozen clay pots in the open flower beds. On 3 September half of the pots were moved into the greenhouse where the temperature was maintained at 8 to 10° throughout the winter. The other pots were left outside.

From July 1957 to June 1958 we determined the resistance of the epiderral cells of the leaf sheath in both groups of plants to elevated temperature and to high hydrostatic pressure (Figures 6 and 7). Heat resistance in July 1957 was about 44°. After some of the plants were moved into the greenhouse, the resistance shown by both groups remained at the former level for 2 weeks. Early in October it began to increase in both groups, but quickly stopped in the greenhouse plants while continuing in the outdoor plants until it reached the usual winter level. By mid-May the heat resistance of the outdoor plants decreased, remaining thereafter at the low summer level.

The pattern of cell resistance to high hydrostatic pressure (Figure 7) proved to be exactly the same. Plants in the greenhouse exhibited only slight increased resistance, whereas in those left in the flower beds the value of the maximum pressure at which the protoplasm continued to move after 5 minutes of action rose from 1100 to 1500 atm. By mid-May resistance dropped to the summer level and then remained there with only trivial fluctuations. This pattern in the greenhouse plants was broken by a jump in the resistance to pressure noted 20 January 1958. This may have been caused, however, by the marked drop in greenhouse temperature when the heating equipment went out of order.

Thus, a comparison of the seasonal changes in cell resistance to elevated temperature and high hydrostatic pressure by plants that overwintered in the open and in the greenhouse shows that the nonspecific resistance noted in grasses in the fall is largely due to the direct effect of low temperature. However, the slight rise in resistance is occasioned by other factors that still await elucidation.

The following experiments also testify to the ability of grass cells to react to cold with a heightening of general resistance. D. glomerata grown in pots was placed in a refrigerator and kept there at different temperatures (from 2.0 to -4.0°) for 9 to 18 hours. Cell resistance to high temperature and high hydrostatic pressure was compared before and after chilling. We discovered that a single chilling scarcely changed the heat resistance. The average increase in resistance was 0.15° in 33 experiments. The same negative result was shown in 15 experiments on resistance to pressure. The chilled plants increased their resistance on the average by 33 atm. We modified the design of the experiments to take cognizance of the data of O. V. Zalenskiy (1955) and M. M. Tyurina (1957) on the significance of photosynthesis in the hardening of plants subjected to night freezing. Plants were placed in a refrigerator in the evening and returned in the morning to the flower bed; the next evening they were again placed in the refrigerator until morning and resistance was determined after they were kept in the light 5-6 hours following the second chilling. The data were compared with the results of the determination made before the first chilling. Table 2 shows that the double chilling raised cell resistance to elevated temperature and to high hydrostatic pressure in all the experiments. This effect was noted both in the summer in plants growing outdoors and in the late fall and early spring in plants taken from the greenhouse. Heightened cell resistance was observed even when a substantial part of the leaf blade was nipped by the double chilling.

Table 2

Effect of Double Chilling on the Resistance of Epidermal Cells in the Sheathing Part of Dactylis glomerata Leaves to High Temperature and High Hydrostatic Pressure

No.	Date	First chilling		Second chilling		Heat resistance (in °C)			Resistance to pressure (in atm.)		
		Temp. (in °C)	Time (in hours)	Temp. (in °C)	Time (in hrs)	Before first chilling	After second chilling	Difference (8-7)	Before first chilling	After second chilling	Difference (11-10)
1	2	3	4	5	6	7	8	9	10	11	12
1	30-VII-57	+2.0	18	+2.0	20	43.8	44.8	+1.0	--	--	--
2	10-VIII-57	0.0	9	-2.5	10	43.8	44.5	+0.7	--	--	--
3	22-VIII-57	-1.5	11	-2.0	10	43.8	44.4	+0.6	--	--	--
4	27-VIII-57	-2.5	15	-3.0	16	43.9	44.8	+0.9	1033	1133	+100
5	11- IX-57	-2.0	15	-2.0	14	43.7	44.7	+1.0	966	1033	+67
6	2- XI-57	-4.0	17	-4.0	17	44.8	45.6	+0.8	1233	1366	+133
7	30- III-58	-4.0	17	-5.0	17	44.3	45.3	+1.0	1200	1433	+233
8	4- VI-58	-2.5	18	-2.5	18	44.6	46.0	+1.4	1033	1333	+300
9	16- VI-58	-2.5	18	-2.5	18	--	--	--	1166	1300	+134
10	19- VI-58	-2.5	18	-2.5	18	44.3	45.8	+1.5	1033	1233	+200
11	19- VI-58	-2.5	18	-2.5	18	--	--	---	1133	1300	+167
12	23- VI-58	-2.5	18	-2.5	18	44.6	46.1	+1.5	1233	1400	+167
13	23- VI-58	-2.5	18	- 2.5	18	--	--	--	1033	1200	+67
						Average		+1.04 ± 0.14	Average		+157 ± 23

Note: Each figure in columns 7, 8, 10, 11--average of three observations in different leaves

Connection Between Increased General Resistance of Grass Cells and Frost Hardening

Thus, grasses exhibit increased cell resistance to high temperature, high hydrostatic pressure, and alcohol in the fall, but this resistance weakens in the spring. It is natural to regard these seasonal changes in sensitivity to completely different agents as proof that the frost hardening of the grass cells is largely due to increased resistance of their protoplasm. This conclusion is supported by data on the sensitivity of D. glomerata cells protected from the cold by being kept in a greenhouse and by experimentally induced heightening of general sensitivity after the plants were chilled twice. Additional confirmation comes from a comparison of curves reflecting seasonal changes in resistance to high temperature and hydrostatic pressure with curves of seasonal changes in cold resistance by the same objects.

Figure 8 (A and B) shows these curves for D. glomerata and E. arenarius. A comparison with Figures 2 and 4-6 suggests that seasonal increases and decreases in cell resistance to cold and other factors occur almost simultaneously. This strengthens our view that changes in resistance to various injurious agents have the same (still undiscovered) causes.

Reversibility of Seasonal Increases in General Cell Resistance

Frost hardening is known to be reversible. In the winter when the plants, or parts of them, are taken from the flower bed and kept in a warm place, cold resistance of the tissues drops. For example, according to I. I. Tumanov (1940), winter wheat Lyutestens 0329 moved into a greenhouse died when kept there for 2-3 days at -15.0° and -13.5°, respectively, whereas plants taken immediately from the cold died at only -20.0°.

M. M. Tyurina (1947) observed increased cold resistance after night frosts in several Pamir plants during the growing period. If the following nights were warm, resistance again dropped after 2-3 days. The literature contains an abundance of similar data on the loss of hardening during the winter. They show that this process takes place in a great variety of plants, although the rate differs markedly in each.

To obtain additional proof of the relationship between heightened frost resistance of cold hardened cells and increased resistance to other injurious factors, we had to find out whether a loss of hardiness results in decreased cell resistance to high temperature, hydrostatic pressure, and alcohol.

D. glomerata were planted in May 1957 in flower pots and grown outdoors. From December 1957 to February 1958 we tested the resistance to heat and high hydrostatic pressure of epidermal cells in specimens taken directly from the garden and at intervals after the plants were moved to the laboratory (room temperature about 18°). Figure 9 presents the curves showing the decrease in over-all resistance with time. It began as soon as the plants were brought indoors, and between the 12th and 18th days cell resistance to the factors being tested reached its summer level (curves I and II). This decrease in over-all cell resistance was accompanied by perceptible growth of the plants.

Hardiness was not lost only in the plant organism as a whole. This phenomenon has been observed frequently in branches of wintering plants brought into a warm place. We noticed it (Aleksandrov and Fel'dman, 1958) even in bits of leaves (about 0.25 cm^2) kept in the laboratory in a box with a daily change of tap water. The decrease in heat resistance of cells in small pieces of leaves kept in water is shown in Figure 9, curve III. The cells of pieces of leaves taken from the plants in the summer and kept in the laboratory usually exhibited a slight growth in resistance to injurious factors. This may well be the cell reaction ot unfavorable conditions following their removal from the plant organism (Aleksandrov and Fel'dman, 1958). Sometimes this increased resistance was scarcely noticeable. As we saw in D. glomerata, bits of leaves taken from plants in the winter react quite differently. This difference in behavior between summer and winter pieces kept in the laboratory is clearly shown in Table 3, which contains the results of experiments to determine the resistance of epidermal cells in the leaf sheath of E. arenarius. In these experiments which were conducted in July and January-February, cell resistance was determined immediately after the leaves were stripped from the plants and after 7 days in the laboratory. We noted a slight rise in resistance to heat and to alcohol in the summer, while resistance to pressure remained unchanged. Similar experiments in the winter showed a clear decline in resistance to all three factors.

Thus, our experiments clearly revealed that tissues transferred in the winter from a cold to a warm place lose their hardiness and become less resistant not only to cold, but to other, completely different factors--heat, high hydrostatic pressure, and alcohol. Hardiness is lost at the cell level, and the process may take place even outside the plant organism.

A comparison of the data set forth above with our earlier data shows that grass cells react in similar fashion to the action of both low and high temperature largely reversible nonspecific rise in resistance. We are thus led to assume that comparable cytological mechanisms underlie these reactions.

Table 3

Changes in the Resistance of Epidermal Cells in the Leaf Sheath of Elymus arenarius When Bits of the Sheath Were Kept in Tap Water in the Laboratory

Resistance	Date (1958)	No. of experiments	Immediately after the piece was taken (A)	Seven days after the piece was kept in water (B)	Difference in resistance of groups A and B
To 5-min. heating	VI	3	45.9°	46.2°	B-A / 0.3°
To 5-min. action of high hydrostatic pressure	VI-VII	9	1089 atm.	1072 atm.	B as % of A 98
To action of 10% alcohol	VI-VII	9	59 min.	98 min.	B as % of A 166
To 5-min. heating	I-II	9	48.8°	45.5°	B - A -3.3°
To 5-min. action of high hydrostatic pressure	I-II	9	1743 atm.	1466 atm.	B as % of A 84
To action of 10% alcohol	I-II	24	94 hrs.	15 hrs.	B as % of A 16

Observations of Seasonal Changes in the Sensitivity of Hepatica Nobilis Cells

Our investigation of seasonal changes in the resistance of epidermal cells in the leaf blade of H. nobilis yielded quite different results from those of the grasses.

Figure 10 shows the results of these observations. Young leaves begin to appear in the spring, at the end of April or beginning of May, depending on the kind of year. Their heat resistance is low. For leaves with blade 5 to 10 cm^2 in size 43° is the maximum temperature after 5-minute

action of which the cytoplasm continues to move. Then, as the leaf grows, the temperature rises to approximately 45.5° and remains at that level throughout the year. There are no indications of increased heat resistance toward winter. Following a favorable winter the plants keep their old leaves until mid-May with more or less damaged blades. Intermingled with them are young leaves. Making a simultaneous test of heat resistance, we found in the former the same magnitude as in the winter -- about 45.5° -- while the latter exhibited much greater sensitivity. This difference was due to the difference in age of the leaves. There were, however, no seasonal differences in heat resistance of epidermal cells in the leaves of this plant.

The data in Figure 10 convinced us that the cells react the same way to hydrostatic pressure. In the spring, as the young leaves develop, cell resistance to pressure rises, but remains almost constant in developed leaves as long as the plant lives regardless of the time of year.

The data set forth above show that, unlike the epidermal cells of grasses, H. nobilis cells exhibit no significant or regular heightening of resistance to heat or to hydrostatic pressure that can be correlated with the onset of cold weather. Nor do the leaves of overwintered leaves become less resistant to these factors in the spring. The cells of young leaves that develop in the spring have low resistance, which increases as the leaves grow.

This raises the question of how cold resistance in the epidermal cells of H. nobilis changes in the course of the year. If frost hardening develops in this species just as it does in grasses, it is due to some other underlying mechanisms that do not involve the heightening of over-all cell resistance. Figure 11 presents data on seasonal changes in the cold resistance of H. nobilis cells. It is apparent that the shape of the curve differs from that of the grasses (Figure 8, A and B). The latter show a distinct and sharp rise in cold resistance in the fall and a drop in the spring. In D. glomerata and E. arenarius cold resistance between December and March is almost 7° higher than between June and August. H. nobilis exhibits between July and August and in December and March the same high cold resistance. The seasonal curve fluctuates somewhat, but not in relation to environmental temperature. The young leaves exhibit only low cold resistance in May; resistance increases markedly as they develop.

Thus, the epidermal cells of H. nobilis scarcely react to the onset of cold with increased resistance. At least there was no indication of it in our tests.

The absence of reactive heightening of resistance in H. nobilis cells following the action of cold led us to pose this question: are these cells generally capable of responding with nonspecific heightened resistance to the influence of injurious factors? To answer the question, we subjected H. nobilis leaves to heat hardening for 18 hours at various temperatures ranging from 28 to 38°. The results are shown in Figure 12. Cell resistance grew as the hardening temperature rose and after hardening at 38° heat resistance increased 1.8° as compared with the control.

It was nonspecific in character, like that of the other plants, since resistance increased not only to heat, but also to high hydrostatic pressure. Halting the movement of protoplasm in the cells subjected to 18 hours of hardening at 35.5° required 180 atm. of pressure more than the cells in the control leaves (average of six experiments).

Thus, expidermal cells in H. nobilis leaves, like the cells of the other plants that we tested, including D. glomerata and E. arenarius (Aleksandrov, 1956; Aleksandrov and Fel'dman, 1958) are able to raise their resistance in response to high temperature. However, unlike the grasses, they exhibit no frost hardening, i.e., reactive rise of resistance after chilling. The level of cold resistance attained by the cells of developing leaves is sufficient by mid summer to assure satisfactory wintering of this species in its natural environment. This does not exclude the possibility of heightened winter and cold resistance by the plant as a whole through other mechanisms that we failed to discover in our experiments.

Discussion

In studying the mechanism whereby some factor injures a living object and analyzing the causes of the object's adaptve increase in resistance to that factor, one of the first problems to be elucidated is the degree of specificity of the phenomenon.

We must first begin by determining the extent to which the injurious action of the factor in question resembles that of factors of different kinds and the way the resultant increase in resistance influences the object's resistance to other actions. The answers to these questions will largely shape the course of subsequent investigation of the entire problem: object - factor.

If the factor is found to be highly specific, the main focus of research should be on the characteristic physical and chemical properties capable of explaining its particular kind of influence on the object. On the other hand, if nonspecific features turn out to be predominant, most of the effort should be aimed at studying the more general properties of the living object.

The literature on frost hardening in plant cells contains some experimental data and numerous opinions to the effect that increased resistance to cold causes the cells to become less sensitive to other influences as well. There is information on the greater resistannce of cold hardened cells to plasmolysis and deplasmolysis (Scarth and Levitt, 1937; Scarth, 1944; Sulakadze, 1949), to ice pressure (Tumanov, 1951), to drowning (Timofeyeva, 1955), to desiccation (Pisek and Larcher, 1954), to drought (Siminovitch, 1940-1941). Illert (1924) called attention to increased heat resistance of Oxalis acetosella toward fall. Sapper (1955) kept flowering specimens of Eranthis hiemalis for 2 days at 10°, 15°, and +4° early in March. Outdoor plants proved to be more heat resistant than those kept in a warm place. According to this investigator, the shoots of Prunus laurocerasus, Hedera helix, and Linaria cymbalaria cut immediately after the winter are more heat resistant than later when warm and rainy weather sets in. Tysdal (1934) found diastasis in extracts of alfalfa to

be more resistant to heat in the winter than in the summer. I. I. Tumanov (1940) thinks that "hardening ... is a universal means of defense by all winter crops against almost all kinds of winter-spring destruction of the plants" (p. 89). Levitt (1951, 1956a, b) is inclined to regard common factors as responsible for resistance to cold, drought, heat, and osmotic loss of water.

Scarth and Levitt (1937), however, report that cold hardened cabbage leaf cells do not exhibit strong resistance to acetic acid and high temperature.

Thus, the literature supports the view that frost hardening is quite nonspecific. However, it is surprising that, despite the many reports on the subject, this highly important problem has not been studied in detail. Our data indicate that seasonal heightening of cell resistance to low temperatures may well be largely nonspecific. With increased cold resistance there is a simultaneous growth of resistance to such different factors as heat, hydrostatic pressure, and alcohol. The nonspecificity of cold hardening that we found in plant cells shows that a study of the phenomenon ought not to be restricted to the action of cold on tissues. It has to be analyzed against the background of the general problem of cell resistance and cell adaptation.

The simultaneous increase in cell resistance to a variety of factors has led us to postulate some vital common link in their injurious action. Otherwise it would be difficult to understand the universality of the hardening process. According to the protein theory of injury and excitation (Nasonov and Aleksandrov, 1940; Nasonov, 1959), the common element is the shift of protoplasmic proteins toward denaturization. The injurious effect of low temperatures on the cell is also attributed by many investigators to denaturization of cell proteins resulting from the intracellular formation of ice (Gorke, 1907; Lidforss, 1907; Schaffnit, 1911; Ullrich and Heber, 1958; Heber, 1958). Levitt in his "mechanical" theory of cell resistance to cold, high temperature, and drought has come fairly close to the denaturization concept. It was demonstrated long ago that with rare exceptions chilling kills cells only if ice forms in the tissues. Of secondary importance in the discussion is the question of what causes denaturization of cell proteins after the tissues are frozen--loss of water, concentration of substances dissolved in cell sap, mechanical action of the ice, or other things.

We can therefore visualize various ways in which cells acquire resistance to cold. Changes reducing intra- or extracellular ice formation may take place in the cells or tissues. The available data indicate that chilling causes intracellular ice to be deposited more slowly after cold hardening (Levitt, 1956 a, b). However, there are no grounds for believing that the defense mechanism against the specific properties of cold as an injurious factor results in heightened cell resistance to completely different influences -- heat, alcohol, or hydrostatic pressure. In those instances where heightened cold resistance correlates with greater resistance to some other factors, it is natural to assume that these defense

mechanisms are linked to the common element underlying the action of the different factors. In accordance with the denaturization theory of frost injury, a general increase in the resistance of cell proteins probably takes place after nonspecific cold hardening. This can be achieved, of course, in various ways.

It is impossible to assume that the increase in resistance here is caused by acceleration of denaturization or resynthesis of protein, as does Allen (1950), for example, in connection with heat resistance of thermophilic bacteria, because there is a general slackening of metabolism at low temperatures. Nevertheless, nonspecific frost hardening, like heat hardening, is associated with protein stabilization due to certain molecular reconstructions or with the appearance of antidenaturizing substances in the cell. Schaffnit was the first to point out that the replacement of labile, macromolecular proteins with simpler and more stable ones might be one of the reasons for increased hardiness. Harwey (1918), Newton (1924), and others backed this view. Siminovitch and Briggs (1949) demonstrated the parallelism between hardiness and the amount of soluble protein, regarding it as a cause and effect relationship. However, there is still no convincing proof of adaptive reconstruction of protoplasmic proteins after hardening.

Many other arguments have been cited in favor of the view that substances functioning as denaturizers may concentrate in the cells. Sugar, whose antidenaturizing action is well known, is one of the more important ones. Lidforss (1907) was the first to assign this role to the sugars accumulating after cold hardening. A large number of studies have dealt with the parallelism between hardening and sugar storage (cf. the summaries of Maksimov, 1913-1952; Tumanova, 1940; Levitt, 1941, 1956). Although a good correlation has often been noted between degree of hardening and amount of sugar, a lack of this correlation has also occurred. Heber (1958) explains this on the grounds that it is not the absolute amount of sugar in the cell that is important, but its concentration in the protoplasm proper. Sugar in vacuoles may have no defensive action. Moreover, the antidenaturizing ability of the various sugars is far from uniform. Finally, cold hardening in plants that are incapable of storing sugar may be ensured by the elaboration of other substances possessing antidenaturizing action. The antidenaturizers specifically include the fatty acids, which are normal products of cellular metabolism (Putnam, 1956).

Many investigators see the defensive action of sugars not in an antidenaturizing effect, but in the elevation of osmotic pressure or reduction of the eutectic point, which may lessen ice formation in the tissues. We do not challenge this possibility, but since cold hardening is associated with nonspecific heightening of cell resistance, it seems to us more probable that stored sugars (or any other substances) function as antidenaturizers.

Thus, the literature and our data indicate that the protein or denaturization theory of injury is a very promising theoretical starting point for an investigation of plant hardiness.

To avoid oversimplification and incorrect conclusions from the above, we must discuss one other problem. The methods of overwintering employed by plants are highly varied (Kozhevnikov, 1950). The possibilities of adaptation to cold are, of course, fewer than in multicellular organisms, but they are by no means limited to a single mechanism. We need mention only the hardiness of plants not storing sugars (Tumanov, 1940) or the numerous cases where increased cold resistance is related to the termination of cell growth, but not in other cases (Tyurina, 1957). We saw above that in some plants the normal movement of protoplasm in the winter is resumed immediately after they are shifted to a warm place, whereas in others it remains stationary. Grass cells exhibit nonspecific heightening of resistance in the winter, but the analogous cells of H. nobilis do not. Consequently, the various forms of cell adaptation must be taken into consideration when studying the winter behavior of plants.

It is clear from this paper that in the three species of grasses resistance to heat, high hydrostatic pressure, and alcohol grows with increased resistance to cold. It is likely that there is also greater resistance to some other agents that we did not investigate. Levitt and Nelson (1942) found that the three kinds of cells in orange peel (epidermal, glandular, and cortical) are arranged in the same order of sensitivity ot high temperature, cold, plasmolysis, and desiccation. It is well known that circumstomatal cells are more resistant than ordinary epidermal cells to a variety of injurious factors. It would be a mistake, however, to conclude from the above that resistance is always "summational" or that cells resistant to one agent are invariably resistant to other agents.

We showed in an earlier paper (Aleksandrov and Fel'dman) that increased cell resistance to heat following individual hardening of a plant and the production of heat resistance in the cells of thermophilic species are phylogenetically assured by various cytophysiologic mechanisms. We find the same thing in resistance to cold. After individual cold hardening there is a well-defined shifting of resistance both to cold and to heat. However, a comparison of heat and cold resistance in various species may not reveal this correspondence. Thus, heat resistance in grasses of tropical origin, e.g., Panicum miliaceum L. and Eleusina indica (l) Gaertn., is much higher than in D. glomerata. The temperature at which the protoplasm ceases to move after 5 minutes of heat is about 49° in the first two plants and about 44° in D. glomerata. At the same time the maximum cold resistance by our criterion is almost identical in all three species -- -8.6° in Panicum miliaceum -7.2° in Eleusina indica, and about -8° in Dactylis glomerata. Thus, the uniform resistance of cells to cold may be combined with heat resistance in very differing degrees.

Findings

L. The resistance of epidermal cells in the leaf sheath of Dactylis glomerata L., Elymus arenarius L., and E. angustus Trin. to heat and high hydrostatic pressure exhibits regular seasonal changes. It is minimal during the growing period (May to Sephember); it rises with the onset of cold

fall weather, remains at a high level throughout the winter, and in April-May again drops to the summer level. The resistance of D. glomerata and E. arenarius cells to ethyl alcohol is also much higher in the winter than in the summer.

2. A comparison of seasonal changes in resistance to heat and to hydrostatic pressure in D. glomerata growing outdoors and in the greenhouse showed that nonspecific heightening of cell resistance in the fall is largely a reaction to a drop in environmental temperature. Chilling may produce nonspecific heightening of resistance even in summer plants .

3. The calendar pattern of changes in cell resistance to heat and high hydrostatic pressure by the three grasses studied coincides almost completely with seasonal changes in cold resistance of these cells.

4. Resistance to heat, hydrostatic pressure, and ethyl alcohol decreases after a loss of hardiness in whole plants transferred in the winter from the open to a warm place or when bits of leaves taken from wintering plants are kept in the laboratory.

5. The cells of the grasses studied react in similar fashion with a reversible and largely nonspecific rise in resistance both to low and to high temperatures.

6. The epidermal cells of the leaf blade of Hepatica nobilis do not exhibit changes in resistance to heat, high hydrostatic pressure, and low temperature that can be correlated with the onset of cold weather.

7. The cells of young H. nobilis leaves have low resistance to these factors in the spring. Their resistance increases as the leaves develop.

8. H. nobilis cells react to superoptimum temperatures with increased resistance to heat and high hydrostatic temperature.

9. An investigation of nonspecific frost hardening in plants on the basis of the protein (denaturization) theory of injury has been shown to be worthwhile (D. N. Nasonov and V. Ya. Aleksandrov).

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THE EFFECT OF PRELIMINARY HEATING ON THE RESISTANCE OF FROG MUSCLE TO THE INJURIOUS ACTION OF HIGH TEMPERATURE AND VARIOUS CHEMICAL AGENTS

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N. A. Shlyakhter
Laboratory of Comparative Cytology,
Institute of Cytology,
Academy of Sciences USSR,
Leningrad

In recent papers V. Ya. Aleksandrov, B. P. Ushakov, and their coworkers showed the coincidence of heat resistance by cells in poikilotherms of the same species from various temperature environments (Aleksandrov, 1952; Ushakov, 1955, 1956, 1958a, b; Dzhamusova, 1958, 1959; Zhirmunskiy and Pisareva, 1958a; Kusakina, 1959; Shlyakhter, 1959). When cold-blooded animals are brought up in high temperature environments, the heat resistance of their cells remains unchanged while the heat resistance of the organism as a whole rises significantly (Zhirmunskiy, 1959; Zhirmunskiy and Shlyakhter, 1959; Svinkin, 1959). There are also no seasonal changes in the heat resistance of ciliated frog and mollusk epithelim (Aleksandrov, 1952; Arronet, 1959). All these data indicate that the heat resistance of cells within the poikilotherm organism as a whole is fairly constant.

References to adaptive changes in isolated tissues after exposure to high temperatures are few. For example, Thörner (1919, 1922) observed adaptation of the frog sciatic nerve to high temperatures, calling it "acclimation." Ye. K. Zhukov (1935) repeated these experiments and confirmed Thörner's findings. Jamada (1924) noted the phenomenon of acclimation in the nonmedullated frog nerve.

V. Y. Aleksandrov (1956) also found increased heat resistance in plant cells after exposure to moderate heat, which he called "hardening." According to Aleksandrov, hardening is a cell reaction to the injurious action of heat.

Our investigation represented an attempt to discover adaptation to high temperature in isolated frog muscle. A study was made of the effect of slight preliminary heat on its resistance to heat injury (38° temperature) and to the injurious action of various chemical agents.

Method

The experiments were performed during the fall and winter on isolated sartorius muscles of grass frogs (Rana temporaria L.). The muscles were kept in Ringer's solution at room temperature for 1.5 to 2 hours after preparation. They were then heated slightly by immersion in Ringer's solution at 34° for 3 to 90 minutes. (Preliminary immersion was only in Ringer's solution at a temperature of 34°, hence the phrases "preliminary immersion," or "preliminary heating.") Immediately afterward the experimental muscles together with the twin controls were exposed to injurious agents -- Ringer's solution at 38° (high temperature), solutions of ethyl

alcohol, quinine, chloral hydrate, calcium chloride and potassium chloride prepared in Ringer's fluid (at 34°), and Ringer's solution diluted fourfold at the same temperature (hypotonia). We judged resistance to the injurious agents by the length of time the muscles retained excitability in the test solutions. The moment of loss of excitability was determined by the lack of contraction in response to irritation by a sinusoidal current, 50 c. frequency (maximum power 2.5 ma). The moment when the experimental muscles could no longer be excited was compared in each series with the time of nonexcitability of the twin control muscles, which was taken as 100%.

The results of the experiments were processed statistically. The degree of probability of the difference (a) between each experimental and corresponding control series of muscles was computed from the Student-Fisher table.

Results

The control muscles kept in Ringer's solution at 38° lost excitability after 15 minutes (average of nine experiments). The experimental muscles exposed to 3 minutes of preliminary heat with subsequent temperature of 38° lost excitability after 17.7 minutes. The delay in onset of nonexcitability was fairly reliable (d = 0.96). (In this work we regarded as reliable the results in which the degree of probability of the difference was at least 0.97.) Following 5 minutes of preliminary immersion and subsequent high temperature, excitability was lost after 20.2 minutes. The increase in time here (/42%) was statistically quite reliable (a = 0.99), i.e., as little as 5 minutes of preliminary heat increased muscle heat resistance. The maximum increase in heat resistance was observed after 15 minutes of preliminary immersion, when it reached 158% as compared with the control (curve a in the figure and Table 1).

Table 1

Effect of Prolonged Preliminary Immersion at 34° on the Resistance of Muscles to a Temperature of 38°

No. of experiments	Time of onset of nonexcitability in the control (in min.)	Time of preliminary immersion (in min.)	Time of onset of nonexcitability in the experimental material (in min.)	$\frac{M_{exp.}-M_{cont.}}{M_{cont}} \times 100\%$	Degree of probability of difference
9	15.0 ± 1.1	3	17.7 ± 1.0	/18	0.96
23	14.2 ± 1.5	5	20.2 ± 1.0	/42	0.99
23	17.1 ± 1.0	10	19.7 ± 1.2	/16	0.95
20	14.9 ± 0.9	15	23.5 ± 0.7	/58	0.99
9	14.6 ± 1.2	20	14.6 ± 1.1	0	--
9	14.7 ± 1.0	30	16.2 ± 0.9	/10	0.88
15	12.7 ± 1.2	40	13.6 ± 1.0	/ 7	0.72
6	13.8 ± 1.4	60	11.3 ± 1.2	-18	0.90
4	16.5 ± 1.0	90	8.0 ± 1.9	-52	0.99

After 20 to 40 minutes of preliminary immersion no further reliable increase in resistance to the heat injury of 38° temperature was recorded, but more prolonged immersion caused a decrease in heat resistance. Thus, after 90 minutes of preliminary heat the time of onset of nonexcitability following the action of high temperature dropped to half that of the control. Consequently, there is a two-phase change in heat resistance during preliminary immersion of the muscles at 34°: first, it rises, reaching the maximum in 15 minutes; then, after longer preliminary heating, it drops and becomes less than in the controls.

We thought it would be interesting to find out whether muscles exposed to preliminary heating show increased resistance to high temperature alone or to other injurious influences as well.

We discovered that brief preliminary immersion at 34° caused changes in resistance to the actionof 5% ethyl alcohol similar to those in resistance to heat (curve b in the figure and Table 2), the maximum rise also taking place after 15 minutes of immersion, whereas the more prolonged action of 34° temperature caused a drop in resistance to the alcohol. Since it turned out that the maximum rise in resistance in these two cases occurred after 15 minutes of preliminary heating (34°), we used just 15 minutes of preliminary heating in the third series of experiments where we studied changes in resistance to certain chemical agents: 0.025 and 0.05% quinine sulphate, 0.5% chloral hydrate, 0.3 and 0.4% potassium chloride, 2% calcium chloride, Ringer's solution diluted fourfold (hypotonia). (The test solutions of these substances, like the 5% alcohol, were at a temperature of 34°. Immersion of the muscles in Ringer's solution at 34% made it necessary to apply the chemical agents after the action of this temperature since it was the only way we could discover how preliminary immersion affected resistance to subsequent chemical injury.) Results of the experiments showing the effect of 15 minutes of preliminary immersion at 34° on resistance to the above-mentioned injurious agents are presented in Table 3. The data indicate that preliminary heating increased resistance to quinine (0.025 and 0.05%), whereas there was no change in resistance to the other agents.

Table 2

Effect of Prolonged Preliminary Immersion at 34% on the Resistance of Muscles to 5% Ethyl Alcohol Heated to 34°

No. of experiments	Time of onset of nonexcitability in the control (in min.)	Time of preliminary immersion (in min.)	Time of onset of nonexcitability in the experimental material (in min.)	$\frac{M_{exp}-M_{cont}}{M_{cont}} \times 100\%$	Degree of probability of differen
7	20.1± 0.5	5	21.4 ± 1.4	+ 6	0.80
12	18.0± 0.7	10	19.7 ± 0.7	+ 9	0.95
18	17.5± 0.3	15	21.1 ± 0.5	+21	0.99
8	18.2± 0.5	20	18.2 ± 0.8	0	--
8	17.0± 0.5	40	14.9 ± 0.6	-12	0.99
12	20.8± 0.9	60	19.0 ± 1.4	- 9	0.86
8	19.0± 0.9	90	15.2 ± 1.4	-20	0.98

Thus, brief preliminary immersion of muscles at 34° increases their resistance not only to high temperature (38°), but also to such chemical substances as alcohol and quinine. It was most pronounced in the case of high temperature (Table 3). Since 15 minutes of immersion at 34° increases resistance to potassium chloride and calcium chloride, hypotonia, and chloral hydrate, the increased resistance must be regarded as being relatively nonspecific in character.

Table 3

Effect of 15 Minutes of Preliminary Immersion at 34° on the Resistance of Sartorius Muscle to Various Injurious Agents

Injurious agent	No. of experiments	Time of onset of nonexcitability (in min.)		$\frac{M_{exp.}-M_{cont}}{M_{cont}} \times 100\%$	Degree of probability of difference
		control	experiment		
38° temperature	21	14.9 ± 0.9	23.5 ± 0.7	+ 58	0.99
Ethyl alcohol 5%	18	17.5 ± 0.3	21.1 ± 0.5	+ 21	0.99
(0.05%	10	16.6 ± 0.4	20.4 ± 1.0	+ 23	0.99
Quinine (					
(0.025%	3	39.3 ± 0.3	45.3 ± 0.7	+ 15	0.99
Potassium 0.3%	14	26.4 ± 2.6	24.5 ± 2.2	- 8	0.72
chloride 0.4%	4	14.1 ± 1.9	14.1 ± 1.9	0	
Hypotonia ($\frac{1}{6}$ Ringer's solution)	8	15.1 ± 1.0	14.0 ± 1.4	- 7	0.72
Calcium chloride 2%	8	11.5 ± 0.3	8.7 ± 0.7	- 24	0.99
Chloral hydrate 0.5%	6	12.7 ± 0.8	12.0 ± 0.5	- 9	0.78

Discussion

"Acclimatizing" a nerve to high temperature, as reported by Thörner, Jamada, and Zhukov, and increased resistance, as in our experiments, involve isolated tissues under somewhat different conditions of elevated temperature. Nevertheless, the same phenomenon may be observed in all the cases -- an increase in heat resistance of the tissue.

In our own experiments we found increased resistance at34°. According to the literature, this temperature is regarded as threshold for the frog sartorius muscle. At this temperature there is an increase in the sorption of vital stains, contracture develops, and the rate of anesthetization of the muscle rises markedly (Butkevich, 1948).

Thus, our data reveal the presence of adaptation to the injury of high temperature, alcohol, and quinine when the temperature action is close to the threshold. T. N. Cherepanova and I. P. Suzdal'skaya (1954) noted a similar phenomenon in their research on the combined action of two salts. They established that liminal and subliminal concentrations of a single stimulant may weaken the toxic effect of strong doses of another stimulant.

After comparing the data of many investigators (Gabrichevskiy, 1887; Gotschlich, 1893; Brodie and Richardson, 1899; Inagaki, 1906; Vrooman, 1907; Mirskiy, 1937; Butkevich,(1948), B. P. Ushakov (1955) pointed out that the threshold of primary heat contraction of the musculature is higher with slow heat than with rapid heat. Thus, in frogs the threshold with slow heat is 37 to 40°, whereas it is 34 to 35° with gradually increased heat. These data indirectly confirm the presence of adaptive processes in muscular tissue following the action of liminal temperatures.

The problem of specificity of acclimation interested Thörner, who unsuccessfully tried to find out whether "acclimatized" nerves develop greater resistance to high temperature and to smothering in nitrogen.

V. Ya. Aleksandrov and N. L. Fel'dman (1958) raised the question of specificity of increased resistance in plant cells. They discovered that leaf cells "hardened" to temperature become more resistant to ethyl alcohol, acetic acid, and high hydrostatic pressure, but not to ammonia. Thus, "hardening" of plant cells proved to be relatively nonspecific. This was also the case with muscles subjected to heat, as in the experiments described above.

The increased resistance noted in our experiments was only the first phase of the muscular reaction to preliminary heating because after prolonged preliminary immersion at 34° resistance to high temperature and alcohol diminished. Comparable phasic changes in resistance to salts were observed in paramecia (L. N. Seravin, 1958).

We concluded from the results of our experiments and the findings of Thörner, Jamada, and Zhukov that adaptation under experimental conditions takes place in many tissues of the organism. B. P. Ushakov (1958a), A. V. Zhirmunskiy and L. N. Pisareva (1958b), and others showed that heat resistance of the animal as a whole is lower than that of its tissues. However, adaptation of the tissues to high temperature occurs with heat alterations that are fatal to the whole organism. Therefore, cellular adaptation to high temperature is evidently impossible as far as the whole organism is concerned.

Findings

1. We studied the effect of preliminary immersion of frog sartorius muscle at 34° of varying duration on its resistance to heat and to diferent chemical agents. Resistance to these injurious agents was evaluated by the time of onset of nonexcitability to a sinusoidal current.

2. The change in muscular resistance to heat during preliminary heating is biphasic in character: brief heating lengthens the time of onset of nonexcitability after 38°, the maximum increase (58%) occurring after 15 minutes. Longer preliminary heating either decreases or has no effect on the time it takes for nonexcitability to develop after 38° temperature.

3. The increased muscular resistance noted was relatively nonspecific. Resistance of preliminarily heated muscles rose not only to heat, but also to ethyl alcohol. It was not found after injury by such agents as potassium chloride, calcium chloride, chloral hydrate, or hypotonia.

4. The shape of the curve showing the relationship between resistance to alcohol and duration of preliminary heating duplicates the curve of changes in heat resistance. The change in resistance to alcohol is biphasic, the maximum (/21%) also occurring after 15 minutes of preliminary heating.

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REPARATIVE EFFECT OF LIGHT ON ANIMAL TISSUES AFTER IRRADIATION WITH SHORT-WAVE ULTRAVIOLET RAYS

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V. L. Levin
Laboratory of Cellular Adaptations,
Institute of Cytology, Academy of Sciences USSR,
Leningrad

The investigation was undertaken in order to find ways of increasing the resistance of tissue cells to ultraviolet rays. The literature contains data on the reparative effect of light on monocellular organisms and sea urchin sexual cells irradiated with ultraviolet rays. However, there is no comparable information on the tissue cells of animals.

Method

A BUV-15 lamp was the source of the ultraviolet rays. Voltage was controlled throughout the experiment and maintained at 127 v. It has been established (Neyshtadt, 1955) that 80 to 90% of the total radiation of this lamp is on the line of 2537 A. We used irradiance (Irradiance was determined with an UFM-5 ultraviolet meter and a baktmeter [?] designed by Golovkin and Nosov. I offer my sincere thanks to Prof. N. A. Golovkin and senior researcher T. A. Sviderskaya for working out the doses and for numerous consultations.) of from 44.0 to 292.5 mw/cm^2. Doses were differentiated by changing the distance from the lamp to the object within the range of 26 to 97 cm and the time of irradiation from 2.5 to 10 minutes.

For experimental material we selected the ciliated epithelium of the pearly mussel (Unio crassus) gills. This tissue was able to survive for a long time explanted; the condition of the epithelium could be determined from observing the cilia. The gill folioles consist of a great many filaments arranged dorsoventrally and separated from one another by canals on the surface, but merging underneath. Three types of ciliated cells may be distinguished in the filaments. Along the central portion stretches a broad band of frontal cells, each of which is covered with small, delicate cilia. Angular or laterofrontal cells are found on both sides of the central band with very large cilia that are thick at the base. On both sides of the filament are lateral cells, each of which has one or more rows of medium-size cilia.

The gill foliole responds to any irritation by slow contraction. The canals then become deeper, the filaments higher and closer together; the laterofrontal cells of the adjacent filaments turn toward one another and close up the canal. This also happens during ultraviolet irradiation. The depth of injury of the various types of ciliated epithelim in the gill varies with the location of the cells with respect to the rays. We therefore studied only the frontal cells, which did not shift during irradiation. We used the epithelium of the ascending lamellae of the internal folioles of the pearly mussel, i.e., the epithelium facing the foot.

Before starting the experiments we cut both internal gill folioles into two halves and distributed the pieces of gills from one mussel into four Petri dishes with a salt solution, (consisting of: NaCl - 1.08 g, KCl - 0.04 g, $CaCl_2$ - 0.05 g, $NaHCO_2$ - 0.3 g per liter of distilled water.) the epithelium of the ascending lamellae facing up. The gills from three to six mussels were placed in each dish. Two of the four dishes were irradiated at a temperature of about 20° and then stored at 5°. One of the irradiated dishes was kept in darkness, while the other was illuminated with a daylight lamp (400 to 500 lux)for the first 3 days after irradiation. The second pair of dishes was not irradiated, but kept under the same conditions -- one in light, the other in darkness. The irradiated and control pieces were examined under the microscope on the 3rd, 7th, 12th, and 17th days after irradiation and later until complete necrosis of the frontal epithelial cells. Following examination the gills were transferred to a new batch of the solution to which 200 units of penicillin per ml were added. Lenses of 12 and 20 power and eyepieces of 15 and 20 power were used. The pieces were not covered with cover glasses.

We rated the condition of the frontal ciliated epithelium in the control and irradiated pieces from the number of surviving and vibrating frontal epithelial cells. Accordingly, we examined 50 to 100 fields of view for each piece and rated the epithelium on the representative scale shown in Table 1.

Table 1

Representative Scale for Rating the Condition of Frontal Ciliated Epithelium

Condition of epithelium	Point
In all fields of view the cilia of all cells vibrate	0
In all fields of view there are individual groups of cells whose cilia do not vibrate	2
In the portions examined the cilia of approximately one fourth of the cells do not vibrate	4
In the portions examined the cilia of approximately one half of the cells do not vibrate	6
In the portions examined the cilia of approximately three fourths of the cells do not vibrate	8
In many fields of view there are groups of cells with vibrating cilia among the dead cells	10
In all fields of view the cilia in none of the cells vibrate	12

Along the ventral edge of the gill foliole extends a broad band whose cells have much higher resistance. In the uninjured gill this outer zone does not differ from the other areas, and even after death of the cells elsewhere on the surface we found here normal movement of the ciliated epithelium of all three types. The same high resistance of the outer zone also occurred after high temperature. We did not take into account the condition of the outer zone when appraising the results of irradiation. Numerical ratings from the various experiments were totaled according to ultraviolet doses and days following irradiation. They were then processed statistically to determine the reliability of the difference between the variants of the experiment. We used the mean values to construct graphs showing the rate of necrosis of irradiated tissue under different conditions. Branchial epithelium from mollusks of different sexes does not react the same way to irradiation and subsequent light (Levin, 1960). Hence, the data in this paper relate only to the more resistant epithelium of females.

Results

In most instances almost all the cilia vibrated in freshly excised gills. The epithelium of the control pieces did not change significantly for 25 to 30 days, during which time only a few cells died. After 30 or 35 days the cells died off more rapidly and by the 55th day the cilia in half the cells on the average were vibrating. However, in some pieces the cilia in a substantial number of frontal epithelial cells were vibrating even up to the 70th day.

No morphological changes developed after irradiation; there was merely accelerated ciliary movement. Some 18 to 30 hours later -- depending on the irradiation dose -- the nuclei of the cells became noticeable and the protoplasm began to get cloudy; larger lumps and vacuoles appeared in the cytoplasm and nucleus. The cells became rounded, lost contact with their neighbors, and separated from the tissue. Besides the cells that remained in their places but which lost ciliary movement, we could see rounded, freely swimming cells with cilia vibrating rhythmically.

Results of our observations are shown in Table 2 and Figures 1-3.

Table 2

Effect of Light on Irradiated Ciliated Branchial Epithelium of the Pearly Mussel

(Mean ratings of the condition of the epithelium are given in points on a representative scale)

Dose in mw. min./cm^2	Conditions of the experiment	Time after irradiation (in days)			
		3	7	12	17
(not irradiated)	Number of experiments	55	45	45	35
	Not illuminated	0.76±0.14	1.14±0.17	1.27±0.15	1.41±0.27
	Illuminated	0.86±0.13	1.02±0.17	1.24±0.15	1.34±0.18
	D ± m_{dif}	0.10±0.19	0.12±0.24	0.03±0.22	0.07±0.27
220	Number of experiments	10	10	10	--
	Not illuminated	0.10±0.10	1.30±1.64	3.70±0.49	--
	Illuminated	0.20±0.08	0.40±0.22	0.80±0.25	--
	D ± m_{dif}	0.10±0.13	0.90±0.68	2.90±0.55	--
333	Number of experiments	17	17	17	22
	Not illuminated	0.24±0.17	2.28±0.30	5.22±0.30	6.86±0.37
	Illuminated	0.18±0.21	0.50±0.19	1.28±0.29	2.18±0.38
	D ± m_{dif}	0.06±0.27	1.78±0.35	3.94±0.42	4.68±0.53
577	Number of experiments	15	15	19	19
	Not illuminated	1.80±0.35	6.80±0.31	8.74±0.21	10.26±0.20
	Illuminated	0.87±0.19	3.87±0.24	6.26±0.27	7.26±0.26
	D ± m_{dif}	0.93±0.40	2.93±0.39	2.48±0.35	3.00±0.33
922	Number of experiments	12	12	12	12
	Not illuminated	3.50±0.50	7.50±0.61	9.42±0.36	10.42±0.34
	Illuminated	2.83±0.52	6.67±0.62	8.33±0.47	9.00±0.3[illegible]
	D ± m_{dif}	0.67±0.72	0.83±0.87	1.09±0.59	1.42±0.[illegible]
1145	Number of experiments	14	14	14	--
	Not illuminated	6.00±0.68	8.80±0.46	9.70±0.50	--
	Illuminated	5.50±0.84	8.40±0.48	9.20±0.47	--
	D ± m_{dif}	0.50±1.40	0.40±0.67	0.50±0.68	--

Table 2 (Cont)

	Time after irradiation (in days)				
	25	31	37	44	52
(not irradiated)	25 2.00±0.23 2.12±0.26 0.12±0.35	25 2.38±0.10 2.52±0.10 0.14±0.14	25 2.96±0.36 3.46±0.47 0.50±0.59	25 4.32±0.63 5.24±0.63 0.92±0.90	14 5.15±0.85 6.14±0.88 0.99±1.22
220	-- -- -- --	-- -- -- --	-- -- -- --	-- -- -- --	-- -- -- --
333	22 9.09±0.37 3.41±0.46 5.68±0.59	22 10.73±0.24 4.81±0.44 5.92±0.50	22 11.59±0.14 6.23±0.51 5.36±0.52	22 12.00 7.32±0.60 4.68±0.60	22 12.00 9.50±0.49 2.50±0.49
577	19 11.79±0.13 9.42±0.29 2.37±0.31	19 12.00 10.68±0.23 1.32±0.23	19 12.00 11.37±0.12 0.63±0.12	-- -- -- --	-- -- -- --
922	12 12.00 10.67±0.45 1.33±0.45	-- -- -- --	-- -- -- --	-- -- -- --	-- -- -- --
1145	14 11.88±0.29 11.20±0.30 0.67±0.42	-- -- -- --	-- -- -- --	-- --- -- --	-- -- -- --

The tables and figures show that all the cells irradiated with ultraviolet rays and kept in darkness died off much faster than the nonirradiated cells; the rate of necrosis of the irradiated cells increased with the size of the dose and ranged from tens of hours to tens of days. The halves of the same gills irradiated at the same time and illuminated for the first 3 days after irradiation with relatively high doses (900 to 1400 mw. min./cm^2 and higher) died at the same rate as those kept in darkness. With smaller doses (200 to 600 mw. min./$cm.^2$) the difference between the condition of the epithelium, kept in darkness and in light became increasingly sharp. There is evidently some threshold radiation dose; with higher doses the reparative processes cannot take place. If the dose is subliminal, however, subsequent illumination activates the reparative processes, as shown by decreased necrosis of individual cells and prolonged survival of the explant

The condition of the explant can also be taken into account when about half the irradiated cells of the frontal ciliated epithelium are stored and continue to function (Table 3).

Table 3

Time of Death of Half the Irradiated Cells (in days) After Five Minutes of Irradiation

Distance from lamp to irradiated piece (in cm)	Dose of ultraviolet rays (in mw.min./cm^2)	Storage condition after irradiation	
		in darkness	in light for first 3 days
30.0	1147	4.0	4.5
34.0	922	5.5	6.5
43.0	577	6.0	11.5
58.0	330	14.5	37.0

The therapeutic effect of light can be very clearly seen in Table 3, which shows that illumination after large doses has no influence on the irradiated epithelium, but that after small doses it more than doubles the survival time of the cells.

The data cited above were obtained from the irradiated and control pieces stored at 5°. We first attempted to ascertain the effect of storage temperature on the course of injury and the reparative processes in irradiated epithelium. Petri dishes containing bits of gills from the same mussels irradiated simultaneously were kept at 5° and 15° either in

darkness throughout or in light for 3 days after irradiation. At 15° hyphae of fungi develop rather rapidly and it is impossible to follow through to the end the fate of explants surviving a fairly long time after illumination. Within as little as 3 days after irradiation the epithelium kept in darkness at 15° was in considerably poorer condition and then died off much more quickly than at 5°. The destructive processes, as was to be expected, proceeded more rapidly at high temperatures. However, even after illumination that strengthened the reparative processes, epithelium kept at 15o (but not at 5°) was nevertheless in poorer condition. On the other hand, the difference between illuminated and nonilluminated epithelium was more pronounced.

The results of these experiments are represented by the curves in Figures 2 and 3, which are constructed the same way as in Figure 1.

Discussion

Our findings correlate well with the published data. I. F. Kovalev (1949) and Kimball (1949) in protozoa, Kelner (1949a) and Novick and Szilord (1949) in bacteria, and I. F. Kovalev (1949) in rotifera found that light repairs injury caused by ultraviolet rays. Subsequent research showed that visible light tends to retard the development of all the changes known to arise after ultraviolet irradiation and even to restore the normal condition of irradiated objects. This phenomenon -- called photoreactivation -- has been studied in detail by I F. Kovalev (1949, 1953, 1958) and L. K. Lozina-Lozinskiy (1959) in protozoa, Dulbecco (1950) in bacteriophages, Bawden and Kleczkowski (1952) in viruses and plant leaves, Kelner (1949b) in fungi, Blum, Robinson, and Loos (1951) and Marshak (1949) in the gametes of sea urchins, and Blum and Matthews (1952) in salamander larvae.

It has been established that photoreactivation is a specific action of visible light, which shows up only after ultraviolet irradiation injury. The following facts are known about the physicochemical mechanism of the phenomenon. Short-wave ultraviolet (λ = 2500 to 2600 Å) causes the maximum injury in biological objects. Long-wave ultraviolet rays and blue-ultraviolet rays of visible light possess photoreactive action (3300 to 4800 Å, according to Dulbecco, 1955) or 2800 to 4700 Å, according to Kovalev (1958). Short-wave ultraviolet rays are largely absorbed by DNA molecules, which are destroyed in the process, injuring normal DNA synthesis in the cell. There is a discharge of acid-soluble phosphorus fractions that are remnants of precursors of DNA molecules from irradiated bacteria kept in darkness (Kelner, 1953; Grundlyand and others, 1958). Photoreactivation can take place following the action of light only a short time after irradiation. Photoreactivation is evidently caused by polymerization of remnan of DNA molecules that failed to escape from the cell and resynthesis of DNA (Grundlyand and others, 1958). On the other hand, the fact that photoreactivation caries with the temperature and that its Q_{10} changes from ~2 in the region of 37° to ~8 in the region of 0° (Dulbecco, 1955) suggests that light affects not the primary photochemical reaction taking place at the moment of ultraviolet action, but the course of the secondary

chemical treactions in the irradiated cell. Latarjet points out in his survey (1954) that light does not act directly on remnants of DNA molecules in irradiated objects, but involves the metabolism of the entire cell in the reparative processes. For example, irradiated sperm can be photoreactivated only after the egg cell is fertilized, and in the case of a virus when the host cell is infected.

V. Ya. Aleksandrov (1952) made a detailed study of the effect of temperature on the development of injury in irradiated cells of infusorians without reference to photoreactivation. A small rise in temperature within the temperature optimum emphasizes the destructive or reparative processes that are predominant at a particular time in the irradiated cell. Temperatures sharply deviating in either direction from the optimum constitute an additional source of injury worsening the condition of infusorians.

We discovered two things in our experiments with the storage of identically irradiated epithelium at various temperature. First, 5° is obviously closer to the temperature optimum of the pearly mussel than 15°; secondly, at these temperatures the destructive processes nevertheless predominate over the reparative processes in irradiated epithelium even after illumination.

Thus, irradiated cells of branchial ciliated epithelium of the pearly mussel show the powerful reparative effect of light. The latter is undoubtedly to be classified with photoreactivation phenomena even though it was impossible after irradiating explanted bits of tissue of multicellular organs to obtain complete recovery of the vital activity of the cells after the period of depression described by other investigators in experiments with infusorians (Kovalev, 1949, 1953, 1958; Lozina-Lozinskiy, 1959).

Findings

1. Ultraviolet rays cause injury, which grows with time, to the branchial ciliated epithelium of the pearly mussel.

2. With moderate irradiation doses (600 mw. min./cm^2) visible light during the first 3 days after irradiation considerably increases the resistance of ciliated epithelial cells to the injurious action of ultraviolet rays.

3. Partial recovery or delay in the appearance of irradiation injury caused by the action of light resembles the phenomenon of photoreactivation described earlier.

4. Experiments involving the storage of epithelium at different temperatures showed that at 15° injury in irradiated epithelial cells develops much more rapidly than at 5° both in darkness and in light.

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RELATIONSHIP OF THE STRUCTURE AND DEVELOPMENT OF THE ORGANISM PRODUCING CHLORTETRACYCLINE (ACTINOMYCES AUREOFACIENS) TO THE SOURCES OF NITROGEN NUTRITION

Pages 707-713

L. N. Kats and A. A. Prokof'yeva-Bel'govskaya
Cytological Group, Selection Laboratory of the All-Union Scientific Research Institute of Antibiotics, Moscow

A number of investigators have recently established that the peculiarities of actinomycetes growth and capacity for antibiotic biosynthesis under the conditions of deep fermentation are closely related to the sources of nitrogen nutrition (Orlova and Verkhovtseva, 1957; Grabovskaya, 1959; Bringberg, Surikova, and Grabovskaya, 1959).

This paper elucidates the relationship of the micromorphology of its culture and developmental peculiarities to the conditions of nitrogen nutrition.

Material and Method

A culture of Actinomyces aureofaciens strain LS-536 served as material for the investigation. Inoculated mycelium was grown on a medium with 2% corn extract (dry weight) and 2.5% starch for 30 hours and introduced in the amount of 3% into a fermentation medium. The control fermentation medium contained corn extract ash and consisted of the following: corn extract ash - 0.03%; NH_4NO_3 - 0.5%, NaCl - 0.2%, $CaCO_3$ - 0.5%, starch - 2.5%, pH - 6.8 to 7.0. The only ingredient in this medium containing nitrogen was NH_4NO_3, which was recommended for this strain by N. V. Orlova and T. P. Verkhovtseva (1957). In the experimental media NH_4NO_3 of the control medium was replaced with soybean,meal, soybean grist, peanut oil cake, urea, and amino acids (alanine, glycocoll, asparaginic acid, leucine; Table 1) and the hydrolyzates of soybean grist and casein in an amount equivalent to the ammonia nitrogen. The urea and amino acids were first dissolved in water, sterilized by passage through a Seitz filter, and placed in the medium just before inoculation. After chalk and other constituents of the nutrient medium (sterilized separately) were put into the experimental flasks, all the media were alkalized in a sterile manner with 20% NaOH or acidified with 2 n. HCL to a pH of 6.8 to 7.0.

Table 1

Amount of Nitrogen From Different Sources in mg per 100 ml of Medium

Ammonia nitrate (Control)	500
Soybean meal	1100
Soybean grist	940
Peanut oil cake	1000
Casein	630
Urea	190
Alanine	560
Glycocoll	470
Asparaginic acid	840
Leucine	825

The innoculated mycelium incubated and fermented in Erlenmyere flasks of 750 ml capacity with 125 ml of medium placed in a rotary shaker (200 rpm) at 28°. Fermentation took 4 days, in some variants about 8 days. During fermentation samples of the culture fluid were tested daily -- for pH potentiometrically, carbohydrate by Shorl's method, weight of dry mycelium by drying at 105° and subsequent weighing, antibiotic activity by the method of diffusion in agar after acidification of the culture fluid with an acid salt to a pH below 2.5. Sporogenous bacilli of the B. subtilis type served as the test organisms. For microscopic examination of the morphological structure of the culture and degree of basophilia of the protoplasm, which indicates its RNA content, we used slightly dried mycelium on a slide fixed with Carnoy's fluid. To study the nuclear elements and determine their DNA content, we stained the mycelium according to Feulgen and with a Giemsa--light green solution (according to Peshkov) after preliminary hydrolysis in 1 n. HCL for 7 minutes at 60°.

Results

The medium with corn extract ash and NH_4NO_3 as the single source of nitrogen, which was the control, exhibited a good growth of the culture and rapid consumption of the carbohydrates. We described in detail the development of the culture in another paper (Katz, 1959). It is characterized by vigorous growth of homogeneous basophilic mycelium in the first phase and by the appearance of a mass of fine hyphae with weakly basophilic cytoplasm and numerous nuclear elements not overloaded with DNA in the second phase. The antibiotic activity of the culture fluid attains a level of 500 units/ml.

In four media where the NH_4NO_3 was replaced with complex products containing chiefly nonhydrolized forms of nitrogen (soybean meal, soybean grist, peanut oil cake, and casein), the culture developed for the most part according to one type, which was in marked contrast with that observed in the control. In the first phase of fermentation, the mycelium proto-

plasm was rich in RNA, which determined its high degree of basophilia as compared with the control and ensured intensive growth of the biomass (Figure 1, 1; Figure 2, 1), the weight of which was 693 mg% on the medium with casein. It was technically difficult to calculate the biomass from the dry weight of the mycelium on the other three media because unused particles of soybean meal, soybean grist, and peanut oil cake settled on the filter. However, the miscroscope showed the development of a large mass of mycelium on these media too after 12 hours of fermentation, accompanied by the growth of very large radiating microcolonies with thick, straight, nonbranching or slightly branching hyphae that frequently formed huge clusters (Figure 1, 1). After 24 hours of growth basophilia of the protoplasm sharply diminished (it began in the control after 12 hours of growth). Numerous, large nuclear elements intensively staining according to Feulgen (Figure 2, 4) could be clearly distinguished in the weakly basophilic cytoplasm (Figure 1, 2 and 3; Figure 2, 2 to 6). This characteristic of the nuclear elements of the mycelium grown on the nutrient medium in these variants revealed their high DNA content and sharply differentiated it from the mycelium cultivated on the control mediu. The ability of the mycelium containing nuclear elements overloaded with DNA to form chlortetracycline markedly decreased (Table 2). Autolysis of the mycelium began after 72 hours of fermentation. The culture fluid was strongly pigmented; its antibiotic activity did not exceed 150 units/ml.

Physiologic Indicators of the Development of an Actinomyces Aureofaciens Culture on Media With Different Sources of Nitrogen Nutrition

Sources of nitrogen nutrition	pH			Biomass (in mg%)			Carbohydrates (in %)			Activity (in units/ml)	
	hours of development										
	24	48	72	24	48	72	24	48	72	48	72
Ammonia nitrate	6.4	6.4	6.8	626	747	566	0.88	0.17	Traces	244	482
Soybean meal	6.4	5.8	6.8	--	--	--	0.94	0.49	0.02	60	67
Soybean grist	6.6	6.5	6.6	--	--	--	0.85	0.51	0.32	95	123
Peanut oil cake	6.6	6.6	6.8	--	--	--	0.80	0.62	0.02	161	133
Casein	6.4	6.4	6.8	253	642	693	0.72	0.60	0.08	150	152
Casein hydrolyzate	6.8	7.6	7.8	551	658	609	0.60	0.09	Traces	88	105
Soybean grist hydrolyzate	6.6	6.7	6.8	560	667	622	0.60	0.32	0.08	113	143
Urea	7.0	7.4	7.6	96	107	--	0.99	0.88	--	0	0
Glycocoll	6.9	7.8	8.0	190	276	500	1.13	0.59	0.08	6	17
Alanine	6.9	7.6	8.1	322	450	788	1.33	0.59	0.02	15	5
Asparaginic acid	7.1	7.7	8.2	123	192	302	1.40	0.65	0.02	17	7
Leucine	6.9	7.1	7.4	115	190	156	1.39	1.36	1.26	Traces	Traces

On media with casein or soybean grist hydrolyzates the culture developed more rapidly than the control, and the micromorphology of the mycelium clearly changed. Large microcolonies developed rapidly in the first phase, sometimes forming a meshwork of mycelium. A substantial part of the mycelial mass was formed within 24 hours of fermentation by basophilic, thickened fragments of hyphae typical of media with high corn extract content rich in the products of protein hydrolysis. Unevenly thickened branching hyphae (Figure 1, 4), which were also characteristic of the medium with glycocoll (cf. below), developed on the medium with the soybean grist hydrolyzate. The mycelial mass grew much faster than on the corresponding media with nonhydrolyzed proteins (Table 2). The weight of the biomass on the medium with casein was 253 mg/% after 24 hours of fermentation, reaching its maximum on the third day (693 mg/%). On the other hand, the medium with the casein hydrolyzate produced a biomass weighing 551 mg/% after 24 hours of fermentation, reaching its maximum (practically the same as the casein medium -- 658 mg/%) on the second day, after which autolysis reduced the weight of the biomass. We observed a similar picture on the medium with the soybean grist hydrolyzate (Figure 1, 5). There was a corresponding acceleration and consumption of carbohydrates (Table 2). Basophilia of the protoplasm in the clearly discernible and fine hyphae decreased quickly. We could ditinguish in the protoplasm, just as in the above-described media with nonhydrolyzed forms of notrogen, numerous nuclear elements (frequently rod-shaped) that stained intensively according to Feulgen (Figure 1, 6). Antibiotic activity of the culture fluid did not exceed 150 units/ml.

Development of the culture on media with urea, asparaginic acid, and leucine was characterized by the formation of isolated microcolonies and extremely low basophilia of the protoplasm, beginning with the earliest hours of development (8 to 12 hours, cf. Figure 3, 1).

This indicated marked suppression under the conditions in the given media of the ability of the protoplasm to synthesize RNA in the first phase of development, which fully matched the strong suppression of culture growth and its weak consumption of carbohydrates (Table 2). The maximum weight of the biomass on the medium with asparaginic acid was reached within 72 hours and only amounted to 302 mg/%, but on the media with urea and leucine growth stopped on the second day, the biomass weighing 100 to 190 mg/%. The mycelium on these media was not uniform: single, short, twisted, segmented, and thickened hyphae developed among weakly developed, fine hyphae. On the second day protoplasm of the hyphae on themedium with urea tended to form vacuoles (Figure 3, 2 and 3). After the 48th hours of growth the culture fluid became alkaline. Nuclear elements at this time were varied in size and stained weakly according to Feulgen. Practically no antibiotic was produced.

On the media with alanine and glycocoll as the sole sources of nitrogen, numerous microcolonies with very thick centers developed within 24 hours of fermentation. The hyphae were short, branching, segmented, angular (Figure 3, 4). The character of the growth changed within 48 hours.

Finer, longer, nonbranching, discontinuous hyphae developed in the culture fluid (Figure 3, 4). The intense basophilea of the protoplasm indicative of its high RNA content remained after 48 hours of fermentation (Figure 3, 5). This was evidently due to the prolonged growth of the culture. The weight of the biomass on the medium with alanine attained its maximum of 788 mg/% on the third day; the growth was less rapid on the medium with glycocoll. The carbohydrates were consumed clowly. The nuclear elements were very large in the weakly basophilic protoplasm of the hyphae during the second phase of development and stained intensively according to Feulgen until fermentation ceased. There was a sharp decrease in the ability to produce the antibiotic. In media where amino acids were the only source of nitrogen, autolysis of the mycelium started after only 6-7 days of growth.

Thus, cytologic investigation of the organism producing chlortetracycline when grown on media with different sources of nitrogen nutrition showed a close relationship between the nature of mycelial growth, development, structure, and ability to produce the antibiotic and the conditions of nitrogen nutrition.

NH_4NO_3 as the only source of nitrogen in the medium ensured the synthesis of RNA and swift growth of homogeneous mycelium in the first phase of fermentation and the appearance in the second phase of a mass of fine hyphae growing lengthwise with weak basophilic cytoplasm and numerous nuclear elements not overloaded with DNA -- typical hyphae capable of producing the antibiotic.

The substitution of complex products in a given medium for NH_4NO_3 -- soybean meal, soybean grist, peanut oil cake, or casein -- stimulated the development of very large microcolonies. The RNA dynamics in the protoplasm was comparable to that of the control but, unlike it, the DNA content in the nuclear elements of the hyphae in the second phase of fermentation rose markedly.

The presence in the medium of complex products containing hydrolyzed forms of nitrogen -- hydrolyzates of soybean grist or casein -- greatly hastened cultural growth and stimulated the development of segmented fragments of hyphae also with high DNA content in the nuclear elements This was deduced from the ability to stain intensively after being processed according to Feulgen.

Substitution of urea or such amino acids as leucine and asparaginic acid for NH_4NO_3 caused severe suppression of RNA synthesis in the protoplasm and inhibition of growth in the first phase of fermentation, whereas in the second phase it resulted only in weak development of fine hyphae.

The presence of alanine or glycocoll in the medium as the only source of nitrogen secured satisfactory growth and stimulated the rapid development of nuclear elements with high DNA content in the second phase of fermentation.

The various types of cultural growths observed on media with different sources of nitrogen may well have been caused not only by a particular form of nitrogen, but also by the specific action on theculture of

the carbohydrates and other constituents introduced into the medium along with the sources of nitrogen under investigation (carbon ring of the amino acids, extractives of the soybean meal, etc.). However, this could not be determined under the conditions of our experiments.

The data obtained in our research on the ability of the chlortetracycline-producing organism to manufacture the antibiotic and the behavioral characteristics of nuclear elements confirmed the earlier findings on Actinomyces rimosus and Actinomyces aureofaciens: the intensive development of nuclear elements with high DNA content was due in all cases to the diminished ability of the mycelium to produce the antibiotic (Prokof'yeva-Bel'govskaya and Popova, 1959; Boretti, Di Marco, Scotti, and Zocchi, 1955).

As pointed out above, the nuclear elements with high DNA content developed rapidly on most of the media with organic sources of nitrogen. This enabled us to study in more detail their structural and behavioral characteristics on media with different sources of nitrogen.

On the media with soybean meal, soybean grist, or peanut oil cake the nuclear elements in the mycelium were large and, in the second phase of development, spherical, localized in the cytoplasm as separate elements more or less evenly spaced from one another. They were occasionally dumbbell-shaped or lay in pairs (Figure 1, 2 and 3), which was seemingly caused by the process of reduplication. On the medium with casein or its hydrolyzate the nuclear elements were very small and arranged in groups of four to six elements each (Figure 2, 3 and 4). On the medium with the soybean grist hydrolyzate the nuclear elements were columnar in form, apparently grouped together inseparably (Figure 1, 6).

This variety of form and arrangement of nuclear elements was obviously due to the fact that it was possible for both the rate of reproduction of the nuclear elements and the rate of growth of the cytoplasmic areas between the nucleotides to vary.

It was established in recent years that one of the major factors determining the DNA content in the nuclear elements of actinomycetes is the phosphorus content of the media (Prokof'yeva-Bel'govskaya and Popova, 1959; Boretti, Di Marco, Scotti, and Zocchi, 1955). This raised the question of whether in this investigation the inorganic phosphorus added as a supplement to the medium along with the nitrogen sources was responsible for the high DNA content of the nuclear elements in the organism producing chlortetracycline.

To answer the question, we checked all the tested variants of the fermentation media for their inorganic phosphorus content. We showed analytically that the phosphorus content of the experimental media ranged from 318 to 6.0 mg/%, which did not correlate with the DNA content of the nuclear elements (Table 3) [See the Figure Appendix for Table 3]. For example, on all three variants of the media--with ammonium nitrate, with leucine, and with glycocoll and an identical amount of phosphorus (3.8 mg/%)--there was weak development of the nuclear elements with low DNA content in the first two cases and rapid development of the nuclear elements heavily overloaded with DNA almost to the end of fermentation in the third

case. These data tend to corroborate the assumption that not only inorganic phosphorus, but also various sources of nitrogen nutrition are the major factor determining DNA content in the nuclear elements of actinomycetes mycelium.

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Findings

1. We investigated the characteristics of the development and micromorphology of the organism producing chlortetracycline (Actinomyces aureofaciens, train LS-536) on media with different cources of nitrogen nutrition (NH_4NO_3, soybean meal, soybean grist, urea, asparaginic acid, leucine, alanine, and glycocoll).

2. The different sources of nitrogen specifically influenced the growth and micromorphology of the culture, RNA synthesis in the protoplasm, structure of nuclear elements and their DNA content.

3. Increased DNA content of the nuclear elements was in all cases caused by the decreased ability of the culture to produce chlortetracycline.

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TEMPERATURE ADAPTATION IN INFUSORIANS. II. CHANGES IN HEAT AND COLD RESISTANCE OF PARAMECIUM CAUDATUM CULTIVATED AT LOW TEMPERATURES

Pages 714-727

Yu. I. Polyanskiy
Laboratory of Cytology of Unicellular Organisms,
Institute of Cytology, Academy of Sciences USSR,
Leningrad

Adaptive modifications are one of the principal ways in which living things adjust to the environment. Protozoa, independent organisms at the cellular level of organization, exhibit wide individual adaptability to a variety of external factors (Serebrovskiy, 1915; Jollos, 1921; Szabuniewicz, 1929; Finley, 1930; Orlova, 1941, 1947; Polyanskiy and Orlova, 1948; Paribok, 1948; Polyanskiy, 1949, 1957, 1958a, b; Seravin, 1958a, 1958b; Sukhanova, 1959). In the first part of this investigation (Polyanskiy, 1957, 1958b) we pointed out the relationship between heat resistance and environmental temperature in paramecia. Heat resistance rises at relatively high temperatures and falls at low temperatures. These changes take place rapidly (over a few hours) and exhibit a regular phasic quality with time. The prolonged action of relatively high temperatures produces lasting modifications in the form of increased heat resistance. The relationship of heat resistance to thermal conditions can be observed over the entire range of the temperature scale -- from almost 0° to 28° or 30°.

In our earlier paper (1957) we did not study the changes with time of heat resistance to comparatively low temperatures nor the relation of infusorians living in various temperature environments to temperature close to freezing -- to 0°. Does the cold resistance of infusorians vary with the preceding temperature regime as in the case of heat resistance?

There are indications in the literature (Yefimov, 1922; Lozina-Lozinskiy, 1948) that 0° is lethal for paramecia and induces characteristic morphological changes -- swelling and assumption of a globular form. For example, in Yefimov's experiments, infusorians transferred to 0° from 18° died within 60 hours. Lozina-Lozinskiy observed the death of paramecia at 0° within 2 days (75% of them died). Futhermore, Yefimov succeeded in prolonging the survival of paramecia at 0° by gradual chilling. His experiments were performed not on clonal material, but on random populations so that the possibility of genotypic differences in cold resistance cannot be ruled out. There are also data on the ability of paramecia to endure brief (measured in minutes) supercooling (Wolfson, 1935; Lozina-Lozinskiy, 1948) to -10°. However, all these experiments were performed without regard for the temperature regime under which the infusorians lived before the start of the experiments.

In general, cold resistance of infusorians has not been adequately studied, and the effect of the previous temperature regime has been scarcely investigated. This problem is of ecological as well as cytophysiological interest. Under natural conditions in the northern latitudes the infusorians live in shallow reservoirs for almost 6 months at about 0°.

This report contains experimental data on the following problems: changes in cold resistance in relation to preceding temperature regime, changes in heat resistance in relation to the cultivation of infusorians at relatively low temperatures, and heat resistance of infusorians cultivated at 0° for a long time.

The material and methods used were the same as those described in the first part of this investigation (Polyanskiy, 1957). The material was clonal. With the brief experiments we used clonal masses of the culture, with the protracted experiments individual lines in microaquaria. We cultivated the paramecia at 0° in test tubes or microaquaria (salt cellars) in large thermos bottles with melting ice. The bottles were kept in a refrigerated cabinet at 4 to 5°. Thus, the ice in the bottles melted very slowly. We changed the ice about once a week.

Cold Resistance of Paramecia (to 0°) after Previous Prolonged Culturing at Various Temperatures

Experiments were run in two variants -- with mass clonal material in small test tubes and with individual lines in salt shakers. The infusorians were placed in a thermos bottle with melting ice to test the survival rate at 0°. The results of the first series of experiments are summed up in Table 1.

Table 1

Survival Rate of Paramecium caudatum at 0° After Preliminary Cultivation at Various Temperatures (mass clonal cultures)

Clone	Temperature of preliminary culturing			
	29°		15 -- 16°	
	Time of preliminary cultivation (in days)	Results	Time of preliminary cultivation (in days)	Results
M'	20	Some infusorians died within 3 hours; all died within 10 hours	39	Some died within 6 hours; none died thereafter
PA	14	About 50% died within 4 hours; all died within 6 hours	33	There were no perceptible changes during the first 12 hours; more than half died within 24 hours; all died in 5 days

Table 2 shows the results of similar experiments with individual lines. Each experiment involved two salt shakers containing six infusorians. Their number and fate were carefully checked for 4 days. The table summarizes the results of the later periods.

The infusorians kept at 5 or 6° exhibited no pathological changes nor were there any deaths when they were shifted to 0° (mass cultures).

Tables 1 and 2 show that cold resistance of infusorians (to 0°) is largely dependent on the preceding temperature conditions. Infusorians kept at 29° invariably die when transferred to 0°. The first deaths may be noted within 3-4 hours and increasingly thereafter. This is a very characteristic picture and has been observed by several other researchers (Yefimov, 1922; Lozina-Lozinskiy, 1948). There is a gradual slackening of movement and swelling until the infusorians become almost globular. The pellicle often cracks and in places tears. No paranecrotic signs can be observed. This picture of death from cold differs markedly from the picture of death from great heat (Polyanskiy, 1957), which is accompanied by paranecrotic changes. Our observations of the death of paramecia at low temperatures do not tally with Greeley's data (1901), which describe dehydration of the cytoplasm in Stentor after exposure to low temperatures.

Pathological changes and death are also observed when infusorians are chilled after being kept at "moderate" temperatures (15 to 18°). However, all these processes take place much more slowly than in infusorians transferred from 29°. There are marked variations in cold resistance. In some clones part of the infusorians transferred from 15-16° did not die at 0° (Table 1, clone M'). Those transferred to 0° from 5 or 6° generally exhibited no pathological changes, did not die, and sometimes divided during the first few days after they were transferred to 0° (Table 2, clone E). However, there were isolated deaths in other clones when transferred to 0° from 5 or 6°.

It is interesting to note the undoubted difference in cold resistance between different clones which, we believe, must be attributed to the genotypic properties of the individual clones. For example, clone P (Table 2) proved to be unusually sensitive to cold. There were few deaths, as compared with the others, among the lines of the clone transferred from 5°. The P lines died much more quickly than the other clones when transferred from 18° or 20° to 0°. Finally, most deaths resulted after transference from 29°. Clone E, on the other hand, showed a different picture, proving to be the most resistant. The "cold" lines of this clone (from 5°) began to divide on the second day of 0°. This clone did not die off even after it was transferred to 0° from 18 or 20° -- three of the 12 infusorians were still alive after a month. Slater (1954) noted similar differences in heat resistance of various lines of Tetrahymena piriformis.

Hairston (1958) found that the various subspecies of Paramecium aurelia reacted in different ways to high and low temperatures. For example, the second subspecies developed better than the others at relatively low temperatures. However, genotypic differences in cold resistance are fairly insignificant as compared with changes in cold resistance due to changes in environmental temperatures.

Table 2

Survival Rate of Paramecium caudatum at 0° After Preliminary Cultivation at Various Temperatures (Individual Cultures in salt shakers)

Period of observation (in days)	from 5°				from 18 to 20°			
	clone S	clone F	clone E	clone P	clone S	clone F	clone E	clone P
1st	all normal	11 alive 1 dead	All alive 2 divided	all normal	all normal	all normal	all normal	6 alive, 6 dead
2nd	"	10 alive 2 dead	14 normal	9 normal 3 dead	9 alive 3 dead	"	"	all dead
3rd	"	10 normal	14 normal	no observation made	9 alive 3 dead	9 normal 2 globular 1 dead	"	--
4th	"	10 normal	14 normal	8 normal 1 dividing 3 dead	7 normal 2 globular	9 normal 1 globular 2 dead	11 normal 1 dead	--
Subsequent development	No deaths observed	no deaths observed	no deaths observed	no deaths observed	all died during the mo.	all died in 14 days	dead	--

Table 2 (Cont)

Period of observation (in days)	from 28 to 29°			
	clone S	clone F	clone E	clone P
1st	2 dead within 4 hours 9 dead by end of day	9 dead by end of day	10 dead by end of day	all dead
2nd	all dead	all dead	all dead	--
3rd	--	--	--	--
4th	--	--	--	--
Subsequent development	--	--	--	--

Our experimental data show that the cold resistance of Paramecium caudatum is largely determined by the previous temperature conditions under which it has lived.

Changes in the Cold Resistance of Infusorians When Transferred From Cold to Warmth

The results of the above-described experiments on the relationship between the cold resistance of paramecia and preceding temperatures raised the question of how swiftly resistance changes when the temperature conditions are modified. To answer the question, we set up two series of experiments -- with mass clonal cultures and with individual lines. The infusorians were first cultivated for a long time (at least 2 weeks) at 5 to 6°. They were then transferred to 29° and their resistance to 0° was tested at different intervals. Infusorians of the same clones from 5 to 6° ("cold control") and from 29° ("warm control") served as controls. The results of the experiments with mass cultures showing changes in cold resistance with time are summed up in Table 3.

Completely analogous results were obtained in experiments with individual lines. Each experiment was performed in two salt cellars with six infusorians placed in each. The results are shown in Table 4.

The results summarized in Tables 3 and 4, which were repeated several times, show that changes in the resistance of infusorians to 0° take place fairly rapidly. However, we noted significant individual differences among them. The initial signs of change in cold resistance appeared in various clones 6 hours after the "cold" infusorians were set in 29°. With longer exposure to 29° cold resistance continued to diminish.

After 72 hours of exposure to 29° the infusorians could no longer be distinguished from the "warm control" (i.e., from those kept for a long period of time at 29°) with respect to cold resistance. Some of them during the intermediate periods (12 to 24 Hours) "feel ill," as expressed in a slackening of movement and in swelling up. However, they eventually recovered and their usual movement returned along with normal body form. A comparison of the rate of change in cold resistance with the rate of change in heat resistance (Polyanskiy, 1957, p 1635, fig. 6) shows that the latter is somewhat more rapid. Statistically valid changes in heat resistance could be observed as early as 2 hours after the temperature was changed, whereas the first signs of change in cold resistance appeared only after 6 hours.

Changes in the Heat Resistance of Infusorians When Transferred from Warmth to Cold

We showed in an earlier report (Polyanskiy, 1957, pp. 1633-4, fig.5) that paramecia cultured for some time at relatively low temperatures (7 or 8°) suffer a marked loss in resistance to lethally high temperatures (40°). However, this process was not studied in detail and the dynamics of changes in heat resistance with time was not elucidated. In the present research we investigated the resistance of Paramecium caudatum to 40° after the clones were transferred to a relatively low temperature (5 to 6°) from room temperature. The tests were made with mass clonal cultures following our regular method (Polyanskiy, 1957, p. 1631). The results are expressed in curves. Figure 1 gives the results of changes in heat resistance by four clones; figure 2 is a composite curve of the same clones.

It is evident from the curves that the changes in heat resistance occurring during the first 4-5 days are far from uniform. There are differences from clone to clone, but the general shape of the curves is more or less the same. Heat resistance decreases very rapidly during the first hours (unusually clear in clones M, M' and 8). It then remains at approximately the same level or even rises somewhat (clones L and 8). Thereafter (60 to 70 hours from the start of chilling) the curve again drops slowly. Thus, the decreased heat resistance of the infusorians when transferred to low temperatures is a process that takes place in brief periods of time measured in a few hours. The character of the curve justifies our speaking of the phase quality of this process with time.

Table 3

Changes in the Resistance of "Cold" Infusorians (from 5 to 6° for 16-18 days) to 0° After Being Transferred to 29° (experiments with mass material in test tubes)

Clone	Control from 5 to 6°	Control from 29°	Resistance to 0° after different periods of transfer of "cold" lines to 29°				
			2 hours	4 hours	6 hours	11-12 hours	24 hours
f	no pathological changes	all died within 3 or 4 hrs	not tested	no pathological changes	within 2 days after transfer partial death began and lasted 2 to 3 days; some of the infusorians remained normal	within 2 days death began, about 2/3 died, the others showed pathological changes (slowing up of movements and phagocytosis); within a week those still alive recuperated	within 5 or 6 hours pathological changes set in (swelling, slowing up of movements); within 24 hours most of the culture died, by the end of 2 weeks all were dead
R	Same	most died within 6 hours; all were dead within 24 hrs	no pathological changes	same	within 2 days a few infusorians died; most of them were alive and normal	not tested	within 6 hours most died; those still alive were distended and scarcely moved; all were dead within 24 hours

Table 3 (Cont)

clone	30 hours	48 hours	72 hours
f	all died within 24 hours	all died within 24 hours	most died within 6 hours; all were dead within 24 hours
R	not tested	same	not tested

Table 4

Changes in the Resistance of "Cold" Infusorians (From 5 to 6° for 20 days) to 0° After Being Transferred to 29° (experiments with individual lines)

Clone	Control from 5 to 6°	Control from 29°	Resistance to 0° after different periods of transfer of "cold" lines to 29°			
			2 hours	4 hours	6 hours	12 hours
1	not set up	all died within 10 hours	no pathological changes	no pathological changes	2 out of 12 died the 4th day, the others were normal; within 2 weeks they began to divide	1 out of 12 died within 2 days, 2 within 3 days, 4 within 4 days, 6 within 6 days; the remaining 6 became ill, recovered in 5 to 6 days, began to divide in 14 days
K	no pathological changes	all died within 24 hours	same	same	3 out of 12 died by the end of the first day; the others were ill for a short time and then recovered	not tested

Table 4 (Cont)

clone	24 hours	48 hours	72 hours
1	all "fell ill"; in 2 weeks 9 died one after the other; the remaining 3 eventually recovered	within 24 hours 6 out of 12 died, 3 more within 3 days; the remaining 3 lived a long time but did not recover normal movement and did not divide	within 24 hours 11 out of 12 died; the single survivor died by the end of the second day
K	3 out of 12 died within 6 hours; all were dead by the end of the first day	not tested	not tested

Changes in the Heat Resistance of Infusorians When Transferred from Cold to Warmth

Experiments were performed on three clones kept at 5 to 6° for a long time (clone 5 for 42 days, clone M for 54 days, clone L for 86 days). The heat resistance of the "cold" lines of all three clones was much lower than that of the control (room temperature 17 to 18°) when tested at a lethal temperature (40°). (It was 8.4%, 9.15%, and 16.5% of the control for clones 5, L, and M, respectively). After the "cold" lines were transferred to room temperature (Figure 3), heat resistance rose steadily and quite rapidly. Within 4 to 5 hours there were statistically valid differences. Within 50 to 90 hours the infusorians acquired the same heat resistance as the control lines.

These results completely resemble those obtained earlier when the infusorians were transferred from room temperature to 29° (Polyanskiy, 1957, p. 1635). We are dealing here essentially with the same phenomenon, but on another part of the temperature curve. There is no basis for assuming phase changes in heat resistance during the first 3-4 days after the infusorians were transferred from 5-6° to room temperature. This wholly agrees with the findings of the previous experiments since phase changes in heat resistance after adaptation to high temperatures occur over much longer periods of time (Polyanskiy, 1957, pp. 1632-1634, figs. 2, 3, 4).

The above-mentioned pattern of changes in heat resistance following transfer from cold to warmth is regularly observed in many clones. There was only one exception (clone PA^1), where prolonged culturing at 5 to 6° merely caused a very slight decrease in resistance. Testing at 40° yielded the following figures: room control 24.0 minutes, "cold" line 19.8 minutes (82.5% of the control). The transfer of cold line PA^1 to room temperature produced a very rapid and sharp increase in heat resistance that amounted to 198% of the control within 14 hours. The reason for this single deviation from the normal pattern is not quite clear. It may be that the original control room line was in a state of partial depression. Chilling brought the infusorians out of the depression, thereby causing a substantial rise in heat resistance.

Prolonged Cultivation of Infusorians at 0°

Our experimental data showed that survival of the paramecia at 0° depended wholly on the preceding temperature regime. The question arose as to how long they could live at 0°. We sought the answer by running experiments in two modifications. The infusorians were transferred from 5-6° to 0° in mass clonal cultures in test tubes (done in the usual way in Lozina-Lozinskiy's medium) and in individual lines in salt shakers, with an accurate record kept of the rate of division. The infusorians grew considerably in all the cultures at 0° as has been repeatedly observed by many observers (Hertwig in 1903 was the first for low temperatures).

The infusorians lived for more than a year at 0° in the mass clonal cultures without exhibiting any signs of depressive changes. Every 2-3 weeks the cultures were transferred to fresh media. Conjugation did not take place. The mass cultures at 0° were set up on 16 May 1958 and are still in good condition at the moment of writing this paper (June 1959).

In individual cultures the rate of division was studied in ten lines. Five of them lived for 6-7 months without exhibiting depression, after which the experiments were discontinued. Five died at different times ranging from 3 to 6 months. Although some of the lines died, the results show that paramecia could survive for a long time and multiply at 0°. The frequency of division at 0° was very low. The interval between two divisions throughout the period of experimentation averaged 17.2 days, varying within broad limits, from 5 to 65 days. In the lines at 0° we did not observe the even rate of division that is characteristic of high temperatures and maintained over long stretches of time. Good examples of the intervals between successive divisions (in days) are line 4--16, 9, 26, 23, 5, 7, 14, 19, 33, 13--and line 5--10, 9, 28, 14, 35, 14, 14, 14.

Thus 0° may well be the temperature at which paramecia can live and multiply for a long time (probably indefinitely). On the other hand, 0°, as we saw above, can also be lethal for the same infusorians, killing them rapidly. The reaction of infusorians to 0° is determined by previous environmental temperatures. Our experimental data suggest that in their natural habitat in reservoirs the paramecia may actively live through the winter.

Heat resistance of Infusorians Cultured for a Long Time at 0°

The facts detailed above show that culturing infusorians at relatively low above 0° temperatures markedly decreases their resistance to heat. We thought it would be worthwhile to investigate the heat resistance of lines of paramecia living at 0° for a long time, i.e., at near freezing temperatures.

Their heat resistance to 40° proved to be very low. The average period of survival of paramecia cultured for a year at 0° yielded the following figures (in min.) at 40°: 3.10 ± 0.05, 3.92 ± 0.01, 3.27 ± 0.003. These figures are 8 to 10 times below those of the lines transferred from room temperature (18 to 20°), which exhibited an average survival at 40° of the order of 25 to 30 minutes.

The relation of paramecia from "zero cultures" (the name which, for the sake of brevity, we shall apply henceforth to cultures living at 0° for a long time) to optimal temperatures for infusorians changed markedly. At 29° we found the normal rapid rate of division and high level of phagocytosis (Reshetnyak, 1952). This temperature turned out to be lethal for the zero cultures. They were transferred to 29° after being kept at room temperature for 15-20 minutes to avoid abrupt temperature changes--the bearing of which on heat resistance was pointed out long ago by Serebrovskiy (1915)--and then placed (in salt cellars or test tubes) in the thermostat at 29°.

We ran a total of six experiments with identical results. Within an hour of exposure to 29° the infusorians in the salt cellars began to die. Their movements slowed down perceptibly, they became deformed--many turning into balls--and died. In the four experiments in salt cellars none of the infusorians was alive after 5 hours. In the test tubes where there were many fewer paramecia, most of them also died within 5-6 hours. Occasional very deformed specimens survived for 3 days, but eventually they too died.

We set up similar experiments involving the transfer of zero cultures to 27°. The picture here was quite mixed. Some of the infusorians (about one-third) died within 24 hours. Others were very deformed, almost becoming balls. They later recovered from their "illness," regained their normal shape and began to multiply. Another group exhibited no significant pathological changes and soon began to divide.

After the zero cultures were transferred to 25°, individual specimens died while others became slightly deformed (swollen). Most of the cultures exhibited no pathological changes and started vigorous agamic multiplication fairly quickly.

It will be noted that the picture of death of infusorians from zero cultures after transfer to 27° and 29° recalls that observed when they are exposed to cold but not to high temperatures (40°). There is body deformation--swelling, globular shape, and breaking of the pellicle. The paranecrotic changes characteristically associated with the action of high letahal temperatures do not occur. It is evident that the unfavorable influence of temperatures of the order of 27 to 29° on zero infusorians is due to impaired physiologic functions of the organisms long adapted to living at low temperatures, but not to the direct influence of temperature on protoplasm proteins.

These facts once again reveal how relative is the concept of "optimum temperatures for infusorians and how much depends on the preceding temperature regime.

Discussion

The material presented in this report shows the very broad range of individual adaptability of Paramecium caudatum to the temperature factor. This adaptability to high temperatures was demonstrated in the first part of the investigation (Polyanskiy 1957). The present paper studied the phenomenon with respect to low above-zero and zero temperatures.

The cold resistance and heat resistance of infusorians both largely depend on environmental temperatures. Changes in either take place comparatively rapidly. Statistically valid shifts are found within a few hours. In this respect infusorians differ considerably from many unicellular organisms. V. Y . Aleksandrov (1952, 1956, 1958), B. P. Ushakov (1955, 1956, 1958), and their co-workers used extensive material to show that heat resistance in the cells of flowering plants and multicellular poikilotherms is a fairly stable quantity for each species. Temperature adaptations here take place not on the cell, but on the "organism" level and are "adaptations of the systemic type" (Ushakov).

A. V. Zhirmunskiy (1959) demonstrated in actinias the possibility of whole organism changes in heat resistance by modifying the environmental temperature. However, there was no concomitant change in the resistance of the cells. N. I. Arronet (1959) noted the lack of seasonal changes in the heat resistance of ciliated epithelium of frogs and lamellibranchiate mollusks at the same time that the resistance of the whole organism showed pronounced seasonal changes. There were only a few cases where it was possible to modify the heat resistance of plant cells by the action of near-lethal temperatures (the phenomenon of "hardening"--Aleksandrov and Fel'dman, 1958). In amphibia the heat resistance of cells can apparently be modified when changed temperature conditions act on ontogenesis (Mikhal'chenko, 1958). These differences in the nature of temperature adaptations of the cells of multicellular organisms and protozoans are probably related to the fact that in protozoans the cell and organism levels of organization coincide so that the "organism" and "cell" forms of adaptation cannot be differentiated (Polyanskiy, 1957). Thus, the possibilities of adaptation for the cells of complex organisms and for cell-organisms are quite different. We can see in this a peculiarity of adaptive evolution in protozoans as compared with multicellular poikilotherms and higher plants.

Individual adaptability (adaptive modifications of organisms) to changing environmental conditions is an important aspect of adaptive evolution based on natural selection. However, the role of cellular elements in the process of individual adaptation differs in protozoans and multicellular organisms.

Findings

1. The cold resistance of Paramecium caudatum to 0° depends on the preceding temperature regime. For infusorians cultured at 29°, 0° is lethal.

2. When infusorians are transferred from a relatively low temperature (5 to 6°) to 29°, changes in cold resistance appear after 6-8 hours and end within 3 days.

3. Heat resistance after the infusorians are transferred from warmth (29°) to cold (5 to 6°) drops rapidly (within a few hours) and is phasic in nature. When transferred from cold to warmth, heat resistance grows swiftly, but is not phasic.

4. Following gradual chilling infusorians can be cultured for a long time at 0° (more than a year in our experiments). At 0° the frequency of division is very low (an average interval of 17.2 days). If cultured for a long time at 0°, the infusorians exhibit very low heat resistance; 29° is a lethal temperature for them.

5. Paramecium caudatum possesses very marked individual adaptability both to relatively high and to low temperatures

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FIGURE APPENDIX

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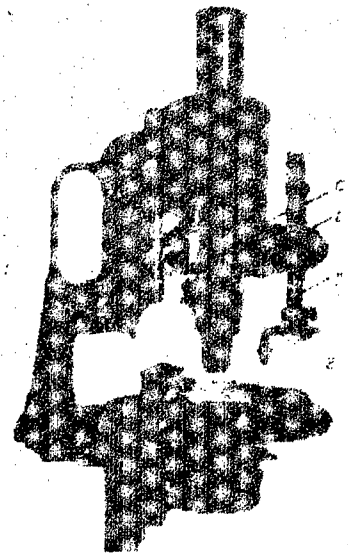

Figure 1. Chekhotin's micromanipulator for mechanical operations on cells.
a. - ring-shaped cap on microscope tube;
b. - rack for lowering of shaft;
c-d. - holder for microinstruments (needles, etc)

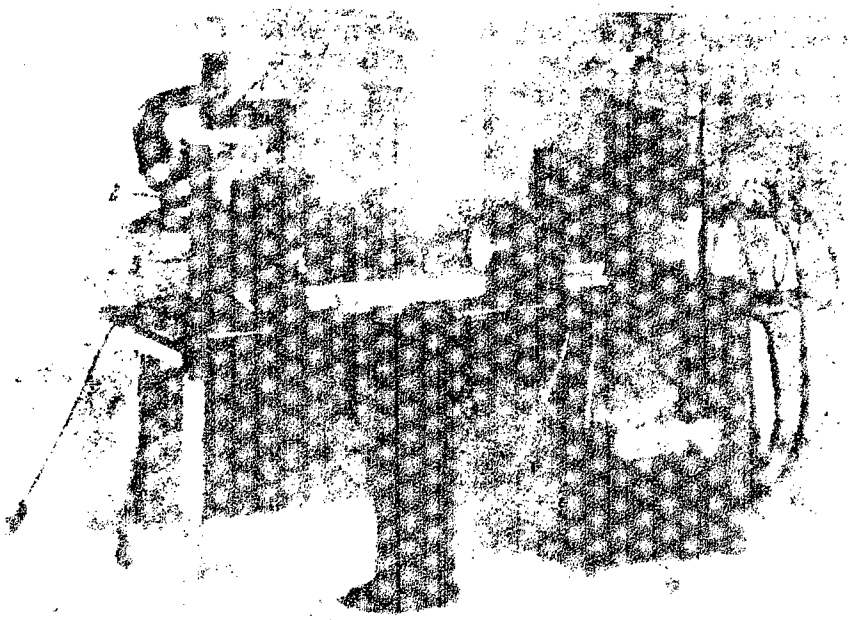

Figure 2. Chalkotin's apparatus for operating on cells by the ultraviolet microbeam method. 1 -- microscope with cap to obtain a dark field; 2 -- quartz prism throwing microbeam upward; 3 -- lamp to illuminate the field of view with a white (ordinary) light; 4 -- optical bench; 5 -- ammeter; 6 -- regulatable diaphragm (microslit); 7 -- stage with quartz prisms (monochrome torus); 8 -- screen to protect eyes from ultraviolet rays; 9 -- quartz lense (collector); 10 -- magnesium electrodes; 11 -- condenser; 12 -- transformer; 13 -- rheostat; 14 -- shaft for movement of electrodes; 15 -- shaft for movement of stage with prisms.

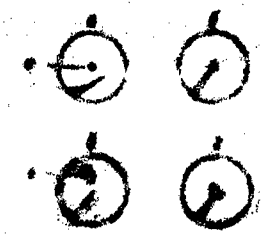

Figure 3. Directing a microbeam on parts of a cell. a, b, c, d -- successive phases of the operation; f -- fluorescent image of the ultraviolet microbeam in cross section on the microscreen; s -- needle indicator in the eyepiece; o -- cell with nucleus.

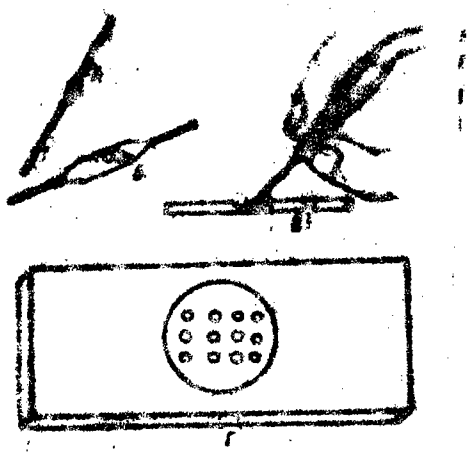

Figure 4. "Microclinic to observe cells after an operation. A -- capillary tube with bacterial culture to feed infusorians in a microchamber; B -- ampul with a sterile medium for the chamber - C -- introduction of food into drop chamber after the cell has been operated on; D -- view of the microchamber arrangement from above.

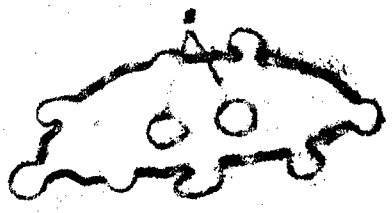

Figure 5. Infusorian Amphileptus pierced by a microbeam at eight points on the body surface with the formation of protuberances.
N -- nucleus

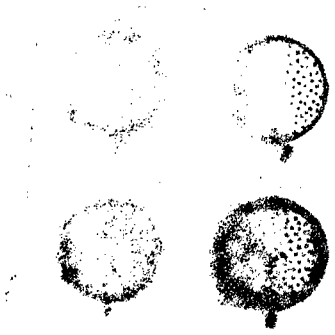

Figure 6. Artificial parthenogenetic activation of sea urchin ovum by ultraviolet micropuncture of a point on the surface. 1 -- irradiation of a point on the surface of the ovum; 2 -- the surface of the ovum flattens at the site of the microphoto puncture; 3 -- the egg membrane forms over the flattened place; 4 -- the membrane spreads over the entire surface of the ovum.

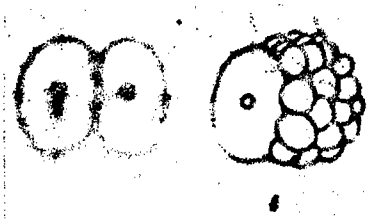

Figure 7. Results on microphoto surgery of the nucleus of one blastomere of a sea urchin ovum. a -- nucleus of the left blastomere pierced by a microbeam (arrow), b -- continuing fission of intact blastomere (on the right).

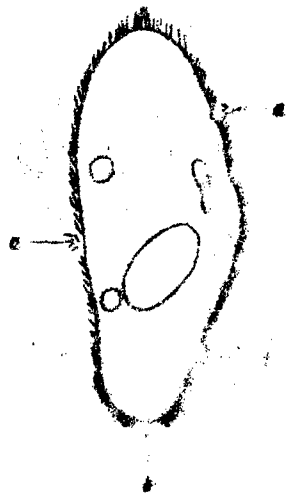

Figure 8. Local microbeam irradiation of the body surface of a paramecium with loss of cilia.
a -- loss of cilia at the point of the surgace pierced;
b -- bare spot on the surface after loss of irradiated cilia;
c -- bare spot on the surgace protruding at the site irradiated.

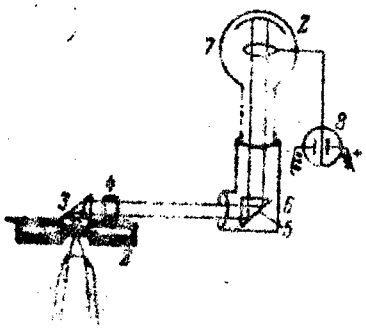

Figure 9. Diagram of an apparatus to measure the difference in absorption of ultraviolet rays by substances in the protoplasm and nucleus. 1 -- quartz lens; 2 -- microcompressor with a cell 3 -- quartz prism; 4 -- quartz lens; 5 -- quartz in tube 6 -- of photoelectric cell 7 --; 8 -- galvanometer.

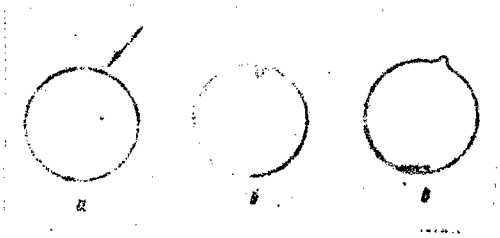

Figure 10. Local changes in permeability of the sea urchin ovum after microbeam irradiation of the surface. a -- point on the ovum surface pierced (arrow); b -- subsequent invagination at this point in a hypertonic medium; c -- protuberance in a hypotonic medium.

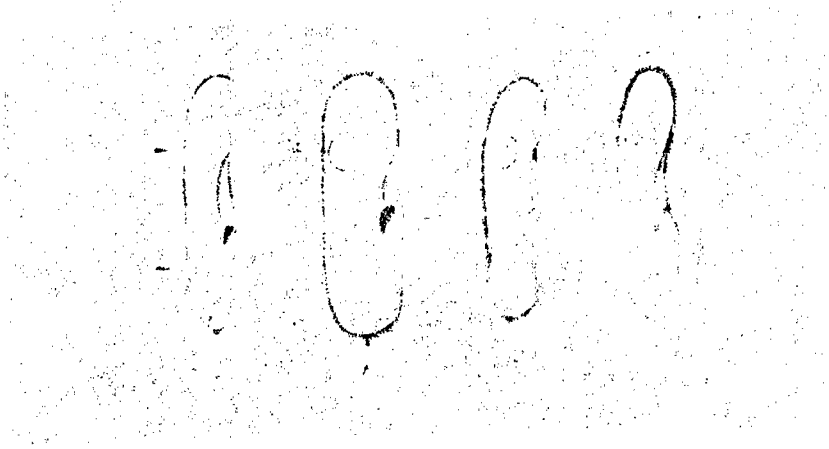

Figure 11. Microbeam operation of contratile vacuoles in a paramecium and treatment of the swelling resulting from the puncture by subsequent irradiation of the cilia in the cytostome.
a, b, c, d -- successive phases of the operation; c' -- cytostome, va - vacuole. The arrows point to the places pierced by the ultraviolet rays.

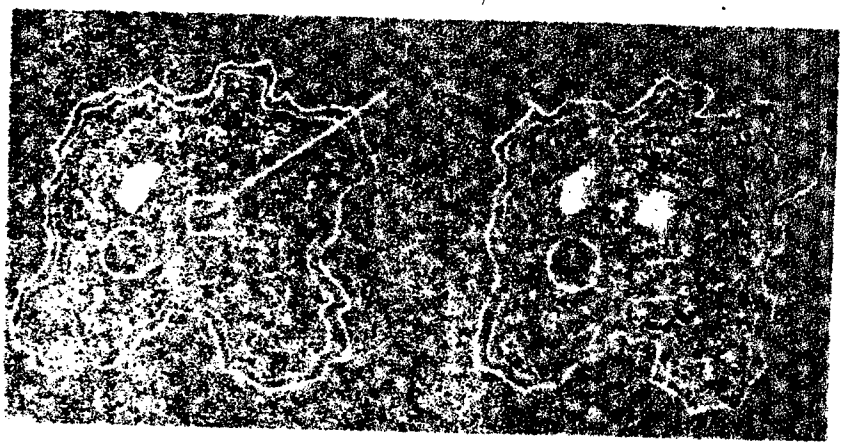

Figure 12. Amoeba proteus in a dark field. Local coagulation of cytoplasm in the cell by ultraviolet microbeam. a -- irradiation of a square area in the protoplasm, b -- coagulation of proteins in the irradiated area.

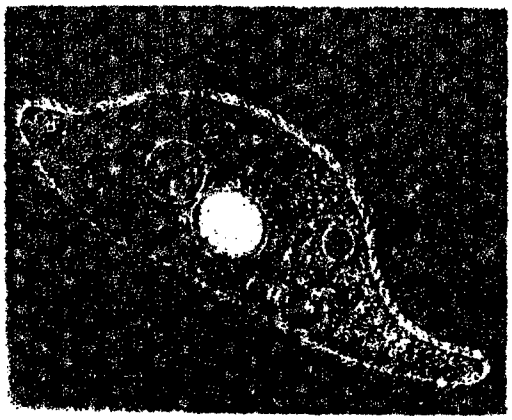

Figure 13. Amphileptus claparedi in a dark field. Irradiation of one of the two nuclei by ultraviolet microbeam.

The lower nucleus has been pierced by the ray and is more transparent in phase contrast.

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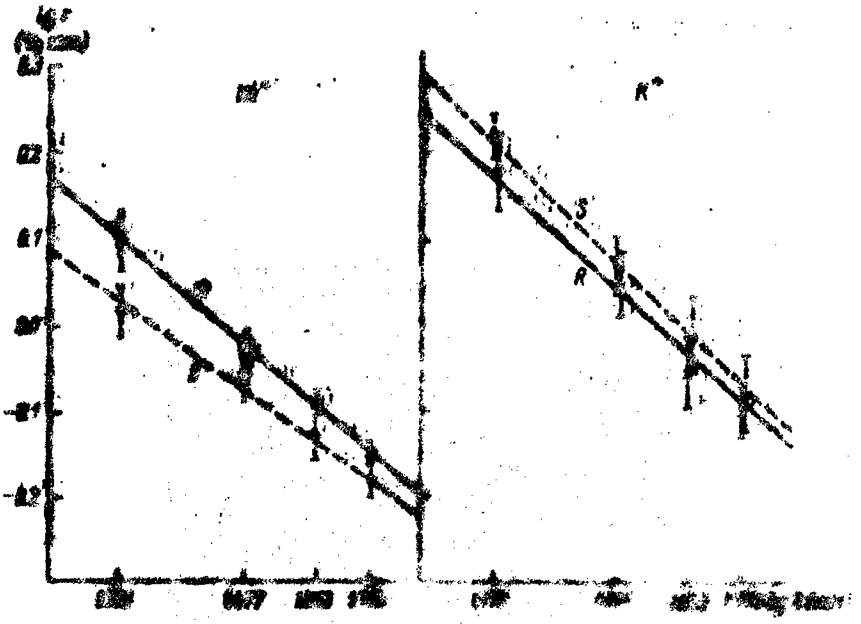

Figure 1. Changes in the diffusion rate of Na^+ and K^+ in extracts from a rat brain in a resting state and after irritation. (For Na^+ in the resting state a = 0.446 and d = 0.440 $\pm$ 0.032, in the excited state, a = 0.19 and d = 0.483 $\pm$ 0.025.) On the x-axis -- lg of time (in min.); on the y-axis -- lg of diffusion (% of ions of the extract diffusing for 1 min.); dots--mean of 8 experiments, vertical lines--standard deviation. The straight lines were constructed from the values computed by the method of least square; R--resting brain, S--excited; slope of the lines expressing changes in the diffusion rate is denoted in the text by d.

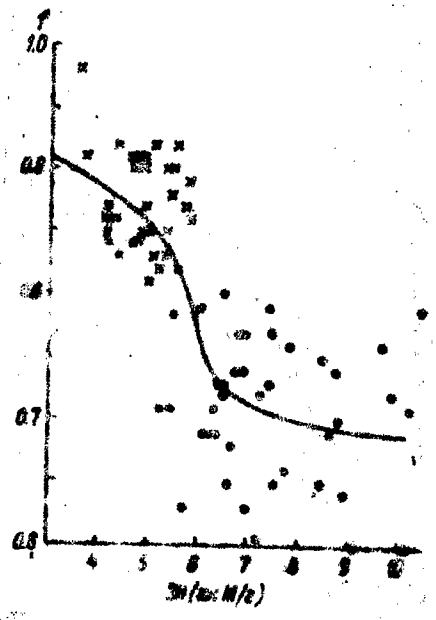

Figure 2. Activity of $Na^{/}$ in extracts from resting and excited rat brain as a function of free Sh-groups.
Along the axis of abscissas - free SH-groups (in uM/g of fresh weight); along the axis of ordinates - activity coefficient (f) of $Na^{/}$. The curve was obtained by plotting the mean values of f between SH 4 to 5 uM, 5 to 6 uM, etc. Crosses -- resting, dots -- excited brain.

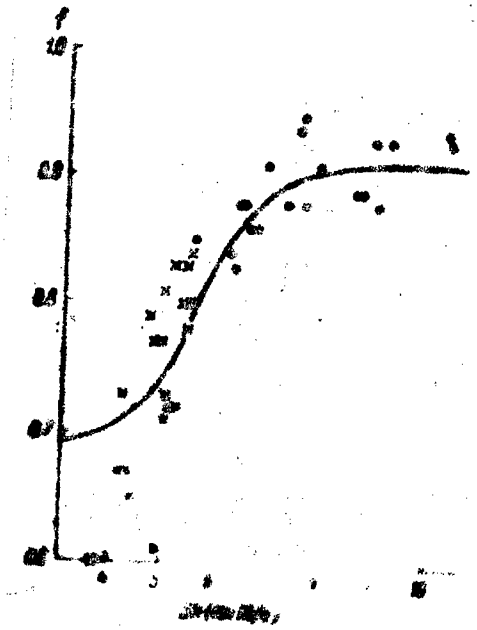

Figure 3. Activity of K^+ ions in extracts from resting and excited rat brain as a function of free SH-groups. Symbols the same as in Figure 2.

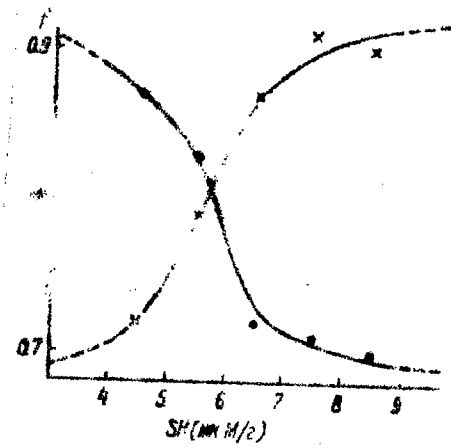

Figure 4. Changes in the activity of Na^+ and K^+ after increased concentration of free SH-groups. Dots - Na^+, crosses - K^+.

Rest

Excitation

Figure 5. Diagram of the absorption hypothesis. K/ is absorbed while the cell is in a resting state. The cell behaves like a potassium electrode and the resting potential like a K/ potential. In an active state the K/ exchanges with the Na/, the cell becoming a sodium electrode, the action potential a Na/ potential.

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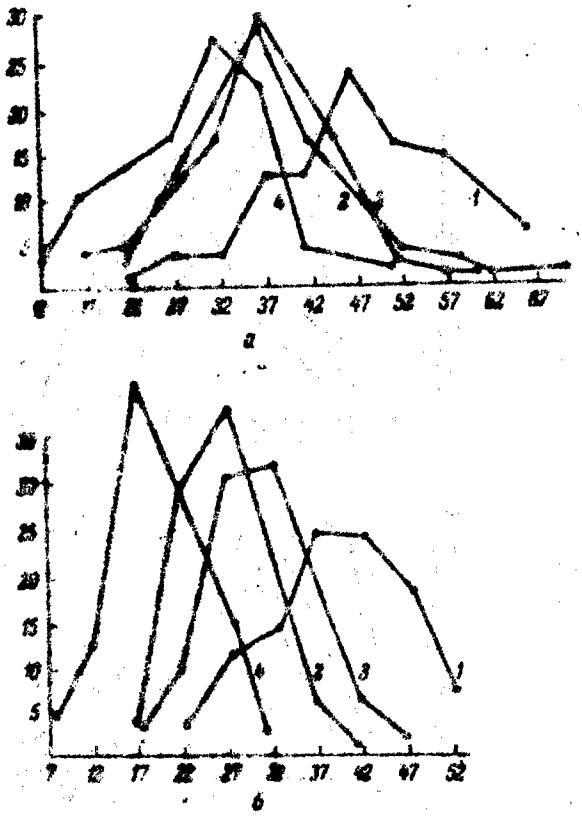

Figure 1. Contraction of mesothelial and mast cell nuclei after the action of 0.25, 0.5, and 1% novocain solutions.
a -- mesothelial cell nuclei, b -- mast cell nuclei. Along the axis of absciasas -- size of nuclei (in conventional units); along the axis of ordinates -- number of nuclei of a given size.
1 -- control (Earl's fluid), 2 -- 0.25% novocain, 3 -- 0.5% novocain, 4 -- 1% novocain.

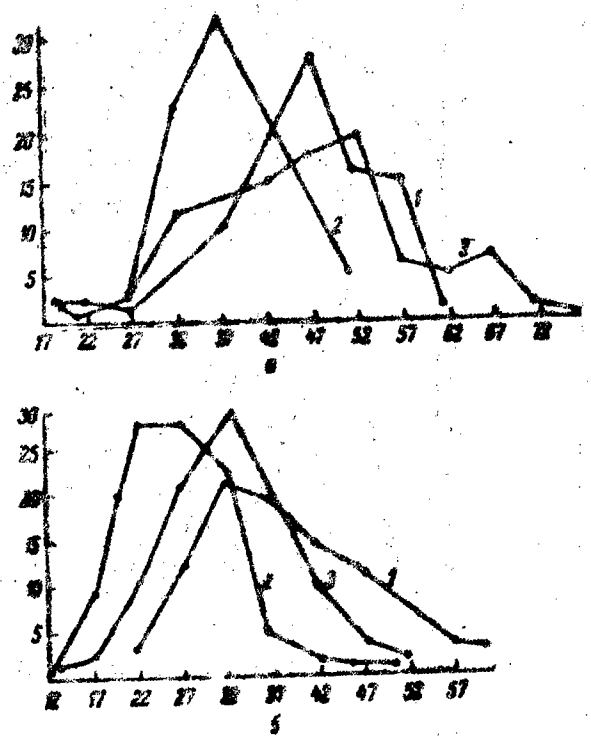

Figure 2. Reversibility of the action of a 0.5% novocain solution on mesothelial and mast cell nuclei. a -- mesothelial cell nuclei, b -- mast cell nuclei. Along the axis of abscissas -- size of nuclei (in conventional units); along the axis of ordinates -- number of nuclei.
1 -- control (Earl's fluid), 2 -- 0.5% novocain, 3 -- reversibility.

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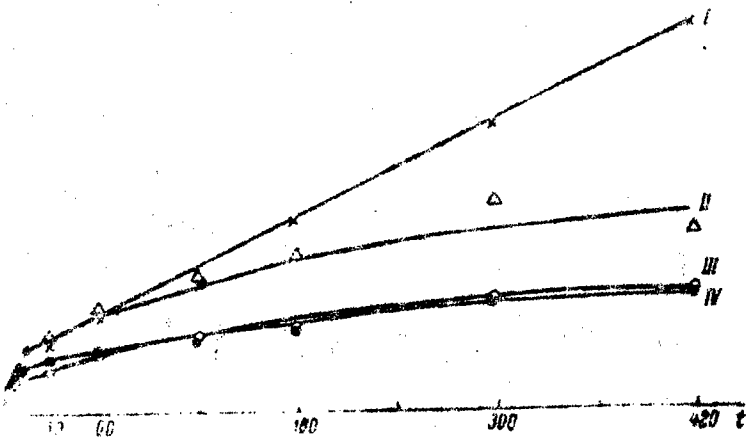

Figure 1. Displacement with time of phosphorus in muscles by labelled phosphorus of the orthophosphate of the surrounding solution under various experimental conditions.
Along the axis of abscissas -- time in min. (t); along the axis of ordinates -- amount of phosphorus in mg per 100 g of wet tissue weight (c).
I - 18°; II - 2°; III - 18°, $NaN_3 = 3.3 \times 10^{-3}$ M; IV - 0°.

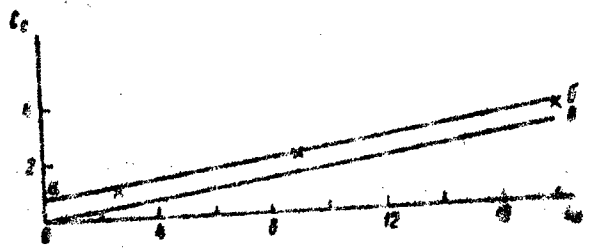

Figure 2. Relationship between phosphorus concentration (in mg%) in muscle fibers (C_c) and its concentration in the surrounding solution (C_s).

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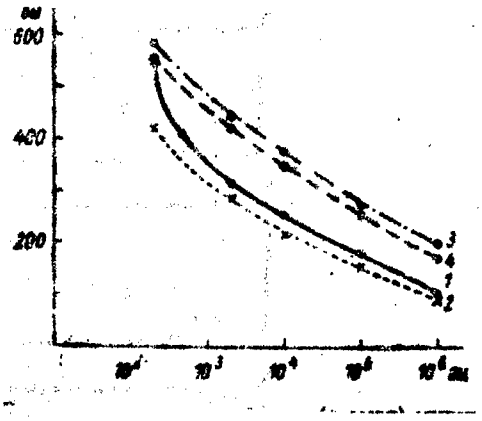

Figure. Relation of resistance (in ohms) of crab and mussel muscles to frequency (in c.) before and after treatment with 4% formalin solution. 1 -- control crab muscle, 2 -- formalinized crab muscle, 3 -- control mussel muscle, 4 -- formalinized mussel muscle.

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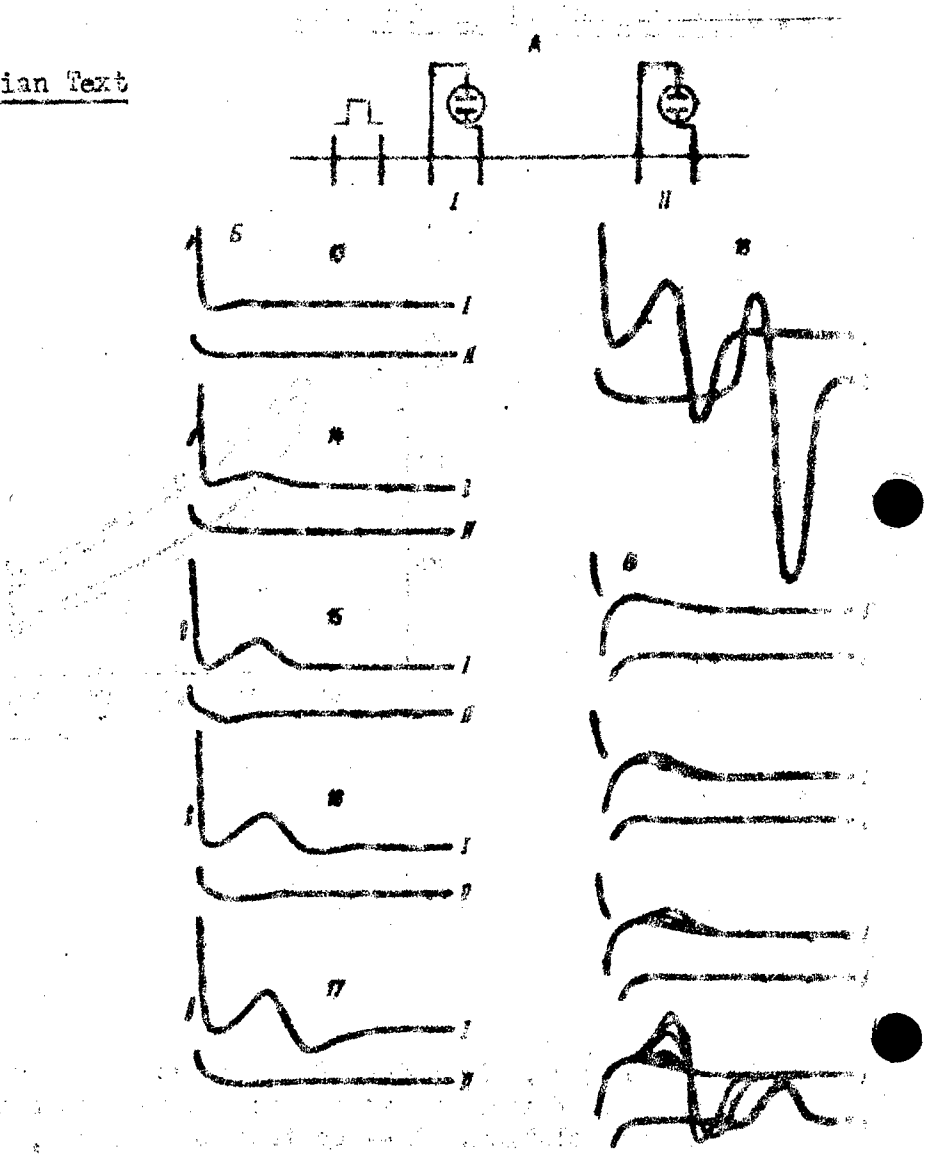

Figure 1. Action potentials after irritation of different intensities.
A -- circuit of irritation and leads of potentials,
B -- experiment of 27 September 1957, C -- experiment of 1 October 1957.
I -- first pair of lead electrodes, II -- second pair of lead electrodes.
Additional explanation in the text.

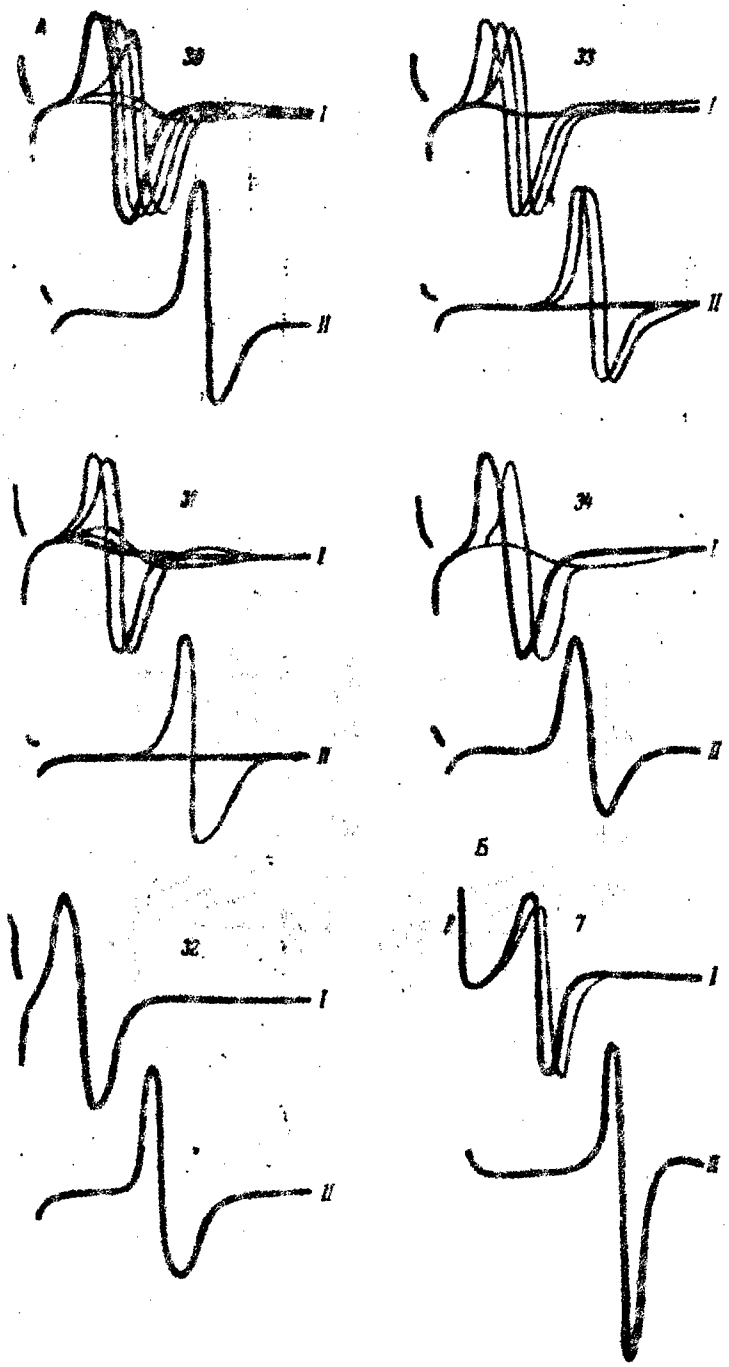

Figure 2. Action potentials after irritation of threshold intensity
A -- experiment of 1 October 1957. B -- experiment of 27 September 1957. I -- lead of first pair of electrodes, II -- lead of second pair of electrodes.

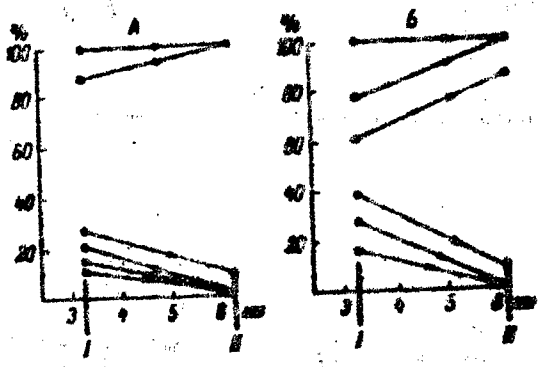

Figure 3. Spatial decrement and increment.
A -- graph of the experiment of 1 October 1957, B -- graph of the experiment of 27 September 1957. I -- lead from the first pair of electrodes, II -- lead from the second pair of electrodes.

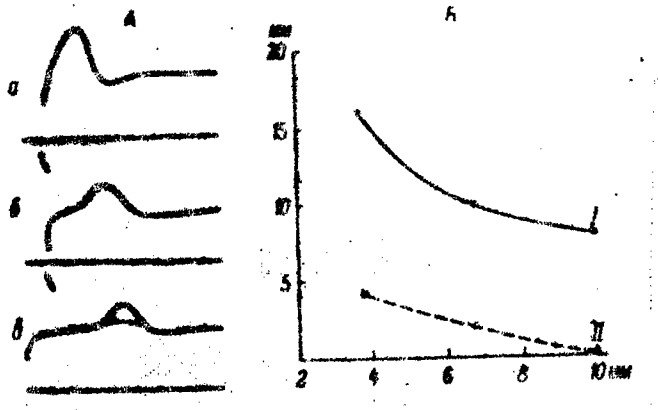

Figure 4. Decremental conduction of excitation with gradual necrosis of the fiber.
A -- changes in magnitude of the action potential depending on the distance between the lead and irritating electrodes (experiment of 1 October 1957): a -- at a distance of 3.7mm, b -- 6.7mm, c -- 9.9 mm, B -- graph based on oscillograms: I -- amplitudes of the first phase of the action potential, II -- amplitudes of the second phase. Along the axis of abscissas -- distance of irritating electrodes from the area of potential lead off; along the axis of ordinates -- potential amplitude (in conventional units).

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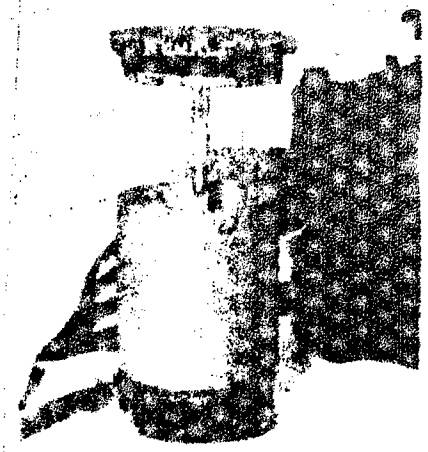

Figure 1. Semiconductof mocrorefrigerator.

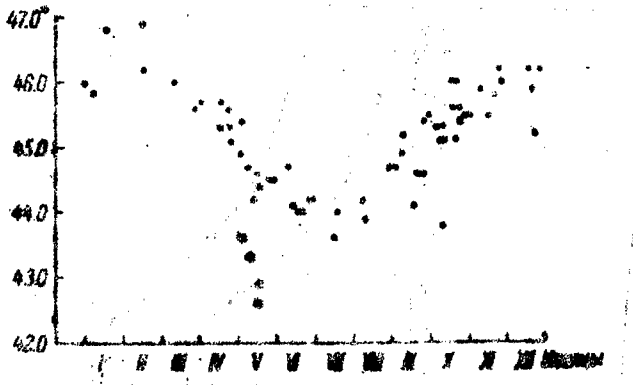

Figure 2. Seasonal changes in heat resistance of Dactylis glomerata cells. Along the axis of ordinates -- maximum temperature. After maintenance of this temperature for a period of five minutes, the protoplasm continued to show movement. The encircled dots were obtained in experiments with new leaves.

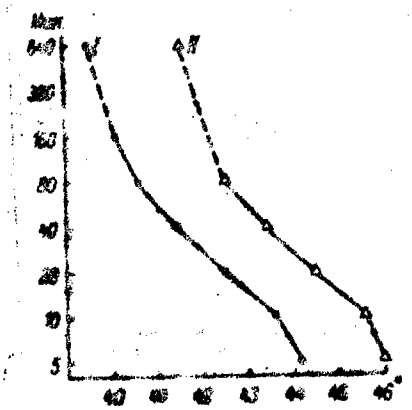

Figure 3. Heat resistance of Dactylis glomerata cells in the summer (I) and in the winter (II).
Along the axis of ordinates -- period of continuation of movement in minutes (logarithmic scale).

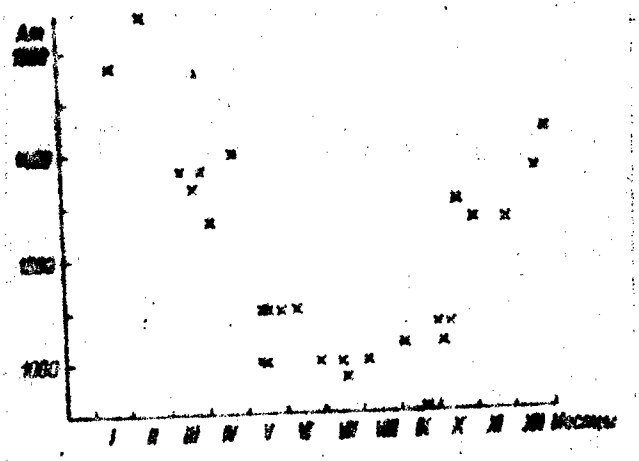

Figure 4. Seasonal changes in the resistance of Dactylis glomerata cells to high hydrostatic pressure.
Along the axis of ordinates -- maximum pressure after 5-minute action of which the protoplasm continued to move.

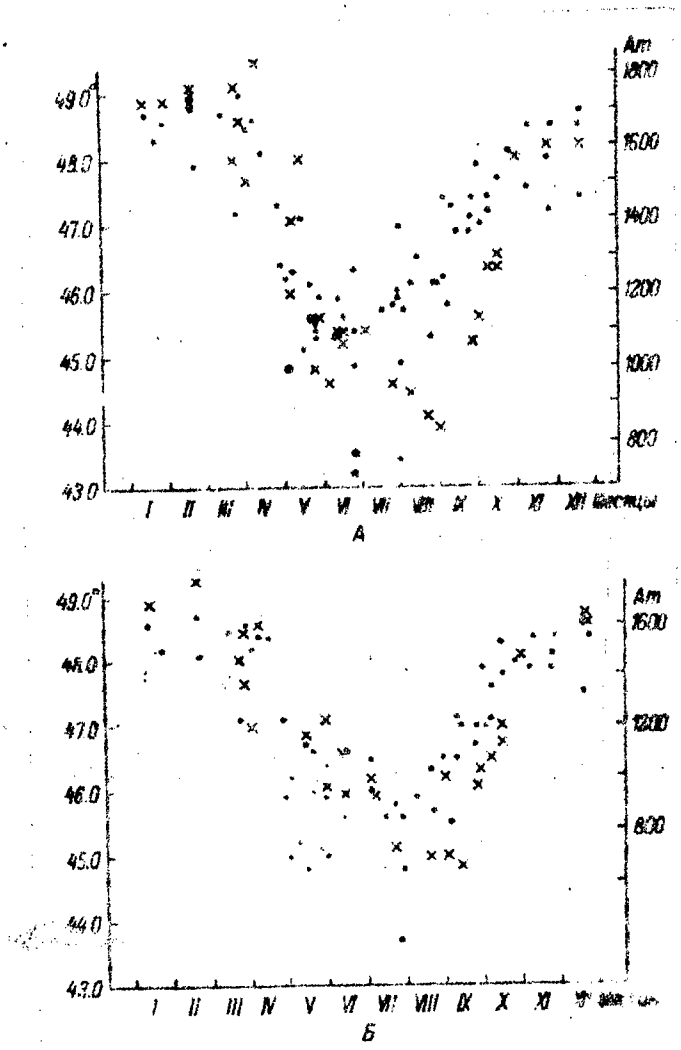

Figure 5. Seasonal changes in resistance to heat (dots) and to high hydrostatic pressure (x's) of Elymus arenarius (A) and E. Angustus (B) cells. Along the axis of ordinates -- maximum temperature (on the left) and maximum pressure (on the right) after 5-minute action of which the protoplasm continued to move. The encircled dots were obtained from new leaves.

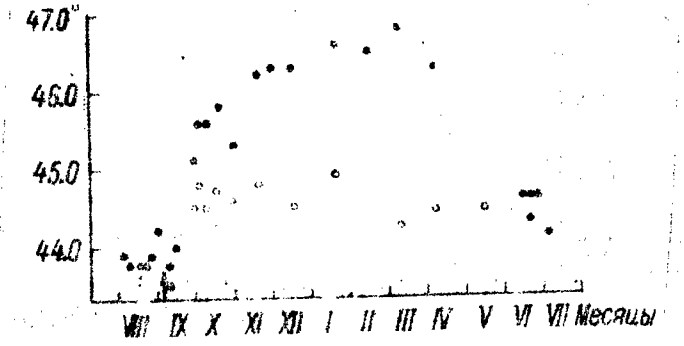

Figure 6. Changes in cell heat resistance of Dactylis glomerata that grow outdoors (dots) and in the greenhouse (circles). Along the axis of ordination -- maximum temperature after 5-minute action of which the protoplasm continued to move. The arrow shows the time when some of the plants were moved from the greenhouse.

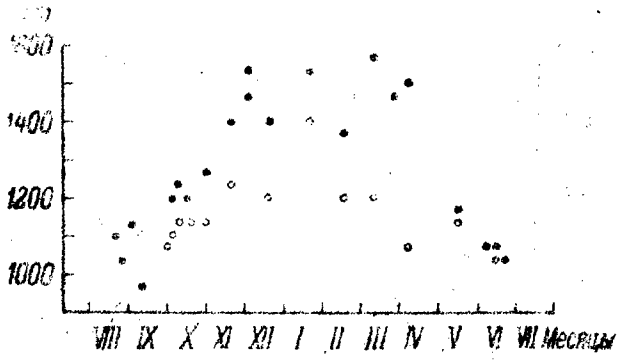

Figure 7. Changes in the cell resistance to high hydrostatic pressure of Dactylis glomerata that grew outdoors (dots) and in the greenhouse (circles).
Along the axis of ordinates -- maximum pressure after 5-minute action of which the protoplasm continued to move.

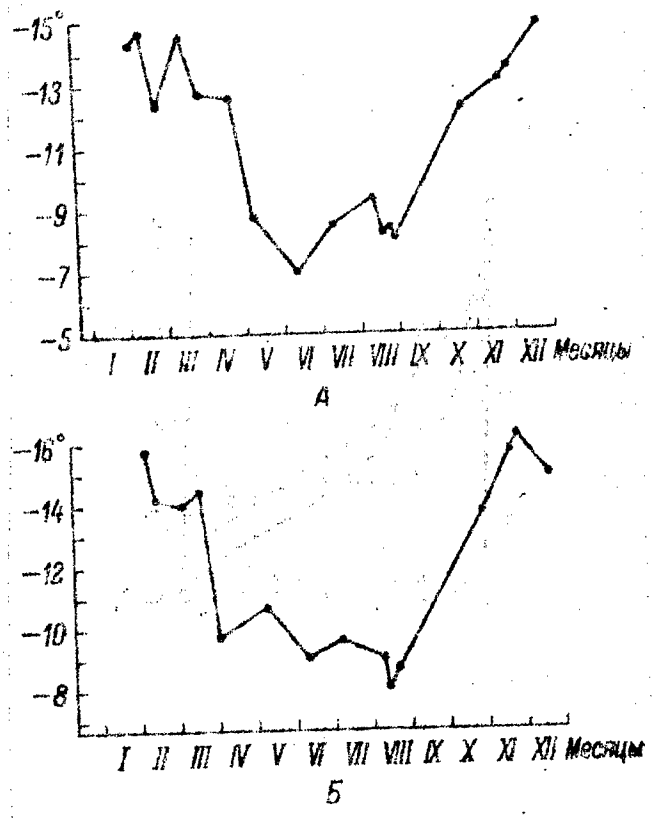

Figure 8. Seasonal changes in cold resistance of Dactylis glomerata (a) and Elymus arenarius (B) cells.
Along the axis of ordinates -- maximum temperature after 5-minute action of which the protoplasm in three pieces of the leaf continued to move.

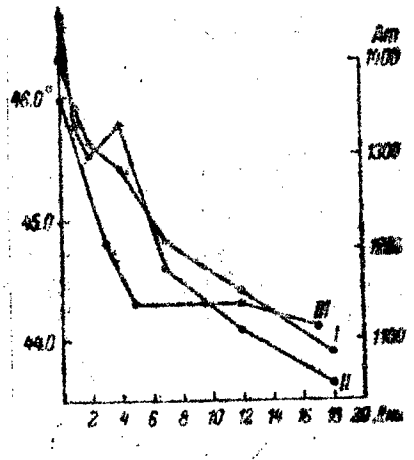

Figure 9. Changes in the risistance to heat (I) and high hydrostatic pressure (II) by cells in Dactylis glomerata leaves after the whole wintering plants were moved to a warm place and changes in the resistance to heat of the same cells when individual portions of the leaves were kept in the place (pomeshcheniye) (III).
Along the axis of ordinates -- maximum temperature (on the left) and maximum pressure (on the right) after 5-minute action of which the protoplasm continued to move; along the axis of abscissas -- days from the time the plants were moved to the warm place.

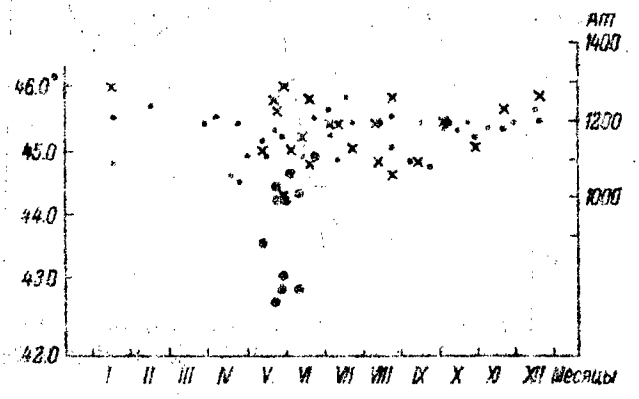

Figure 10. Seasonal changes in the resistance to heat (dots) and to high hydrostatic pressure (x's) of Hepatica nobilis. Along the axis of ordinates -- maximum temperature (on the left) and maximum pressure (on the right) after 5-minute action of which the protoplasm continued to move. The encircled dots were obtained from new leaves.

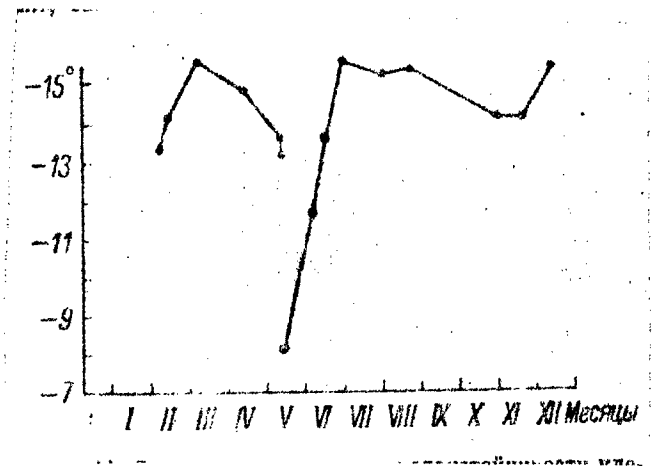

Figure 11. Seasonal changes in the cold resistance of Hepatica nobilis cells. Along the axis of ordinates -- minimum temperature after the 5-minute action of which protoplasm continued to move in three places of leaf.

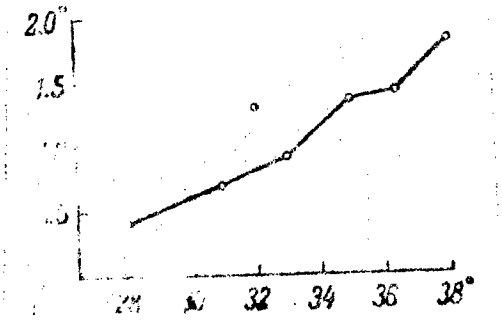

Figure 12. Increase in heat resistance of Hepatic nobilis cells in relation to the temperature at which they became hardened. Along the axis of abscissas -- hardening temperature; along the axis of ordinates -- rise of temperature halting the movement of protoplasm after 5 minutes of heat as compared with the control.

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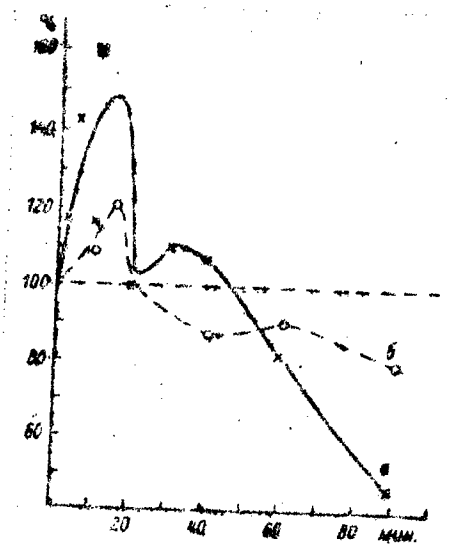

Figure. Curves of changes in muscle resistance to heat injury and ethyl alcohol injury.
a -- injury by 38° temperature, b -- injury by 5% alcohol at a temperature of 34°. Along the axis of abscissas -- time of preliminary immersion of muscles at a temperature of 34° (in min.); along the axis of ordinates -- changes in the time of loss of excitability as % of the control taken as 100%.

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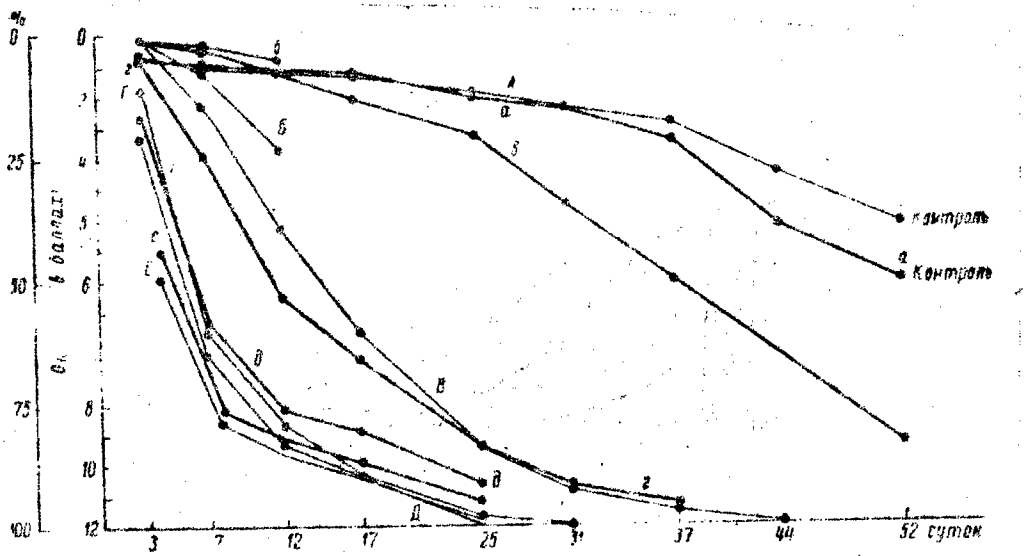

Figure 1. Curves reflecting necrosis of ciliated bronchial epithelium of the pearly mussel when kept in darkness and in light after irradiation with different ultraviolet doses.
Along the axis of abscissas - time after irradiation; along the axis of ordinates -- percent of dead cells and rating of condition of epithelium. The "light" and "dark" variants of the same experiment are designated with the same letters ("dark" - capital letters, "light" - lower case letters): A and a - nonirradiated epithelium; B and b - irradiation dose (in mw. min./cm^2) 220; C and c -- 333; D and d -- 577; E and e -- 1147.

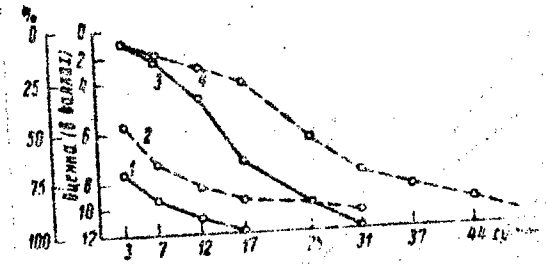

Figure 2. Curves showing necrosis of cells of ciliated bronchial epithelium of the pearly mussel at 5^0 and 15^0 after irradiation with a dose of 488 mv. min./cm^2.

Along the axis of abscissas -- time after irradiation; along the axis of ordinates -- percent of dead cells and rating of condition of epithelium. 1 -- epithelium kept at 15^0 in darkness, 2 -- epithelium kept at 15^0 in light, 3 -- epithelium kept at 5^0 in darkness, 4 -- epithelium kept at 5^0 in light.

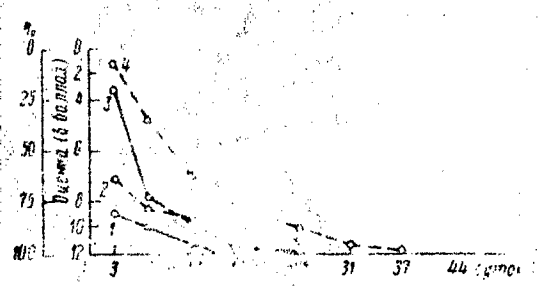

Figure 3. Necrosis of cells of cilliated branchial epithelium of the pearly mussel at 5^0 and 15^0 after irradiation with a dose of 577 mw. min./cm^2. Designations the same as in Figure 2.

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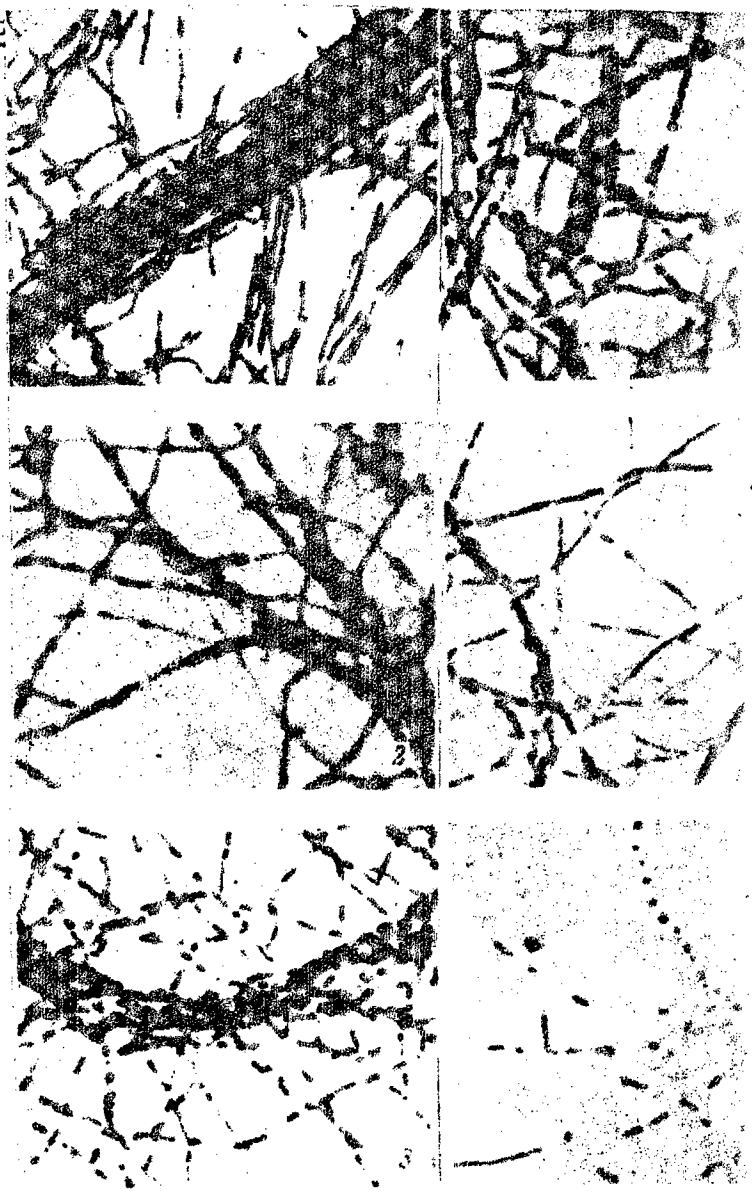

Figure 1. Development of a culture of Actinomyces aureofaciens, strain LS-53Ø, on media with soybean grist and its hydrolyzate. Fixation with Carnoy's fluid. Microphot. obj. 90x ap 1.3, eyepiece 10x, magn. 2000x.

1, 2, 3, --medium with soybean meal: 1 -- 24 hours of growth, staining with methyl blue; 2 -- 48 hours of growth, staining with methyl blue; 3 -- 48 hours of growth, staining with Giemsa -- light green. 4, 5, 6 -- medium with hydrolysate of soybean meal: 4 -- 24 hours of growth staining with methyl blue; 5 -- 48 hours of growth, staining with methyl blue; 6 -- 48 hours of growth, staining according to Feulgen.

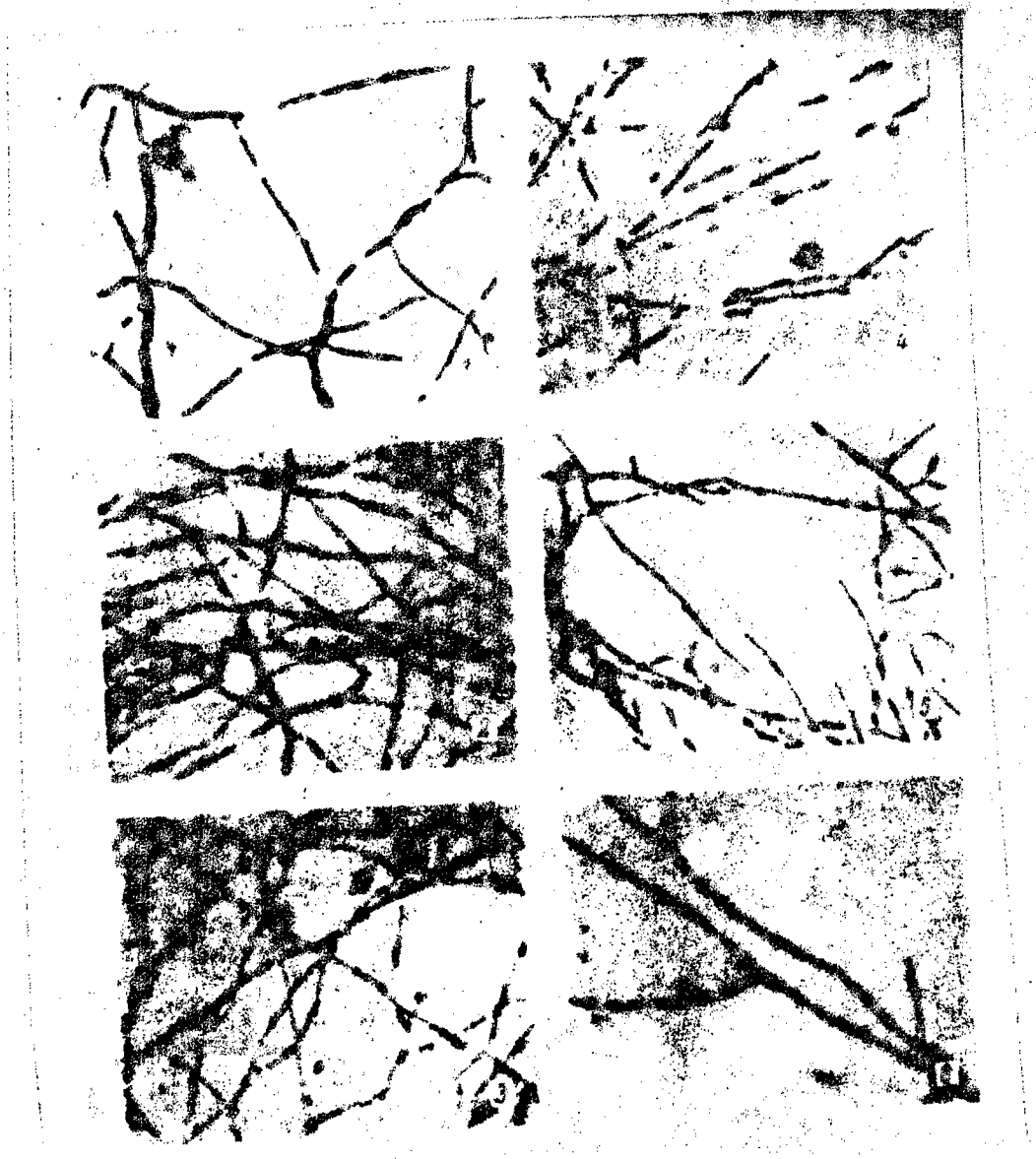

Figure 2. Development of a culture of Actinomyces aureofaciens, strain LS-536, on media with casein and soybean meal. Fixation with Carnoy's fluid. Microphot., obj. 90x a;. 1. 3, eyepiece 10x, magn. 2000x.
1, 2, 3, 4 -- medium with casein: 1 -- 24 hours or growth, staining with methyl blue; 2 -- 48 hours of growth, staining with methyl blue; 3 -- 48 hours of growth, staining with Giemsa -- light green; 4 -- 48 hours of growth, staining according to Feulgen; 5, 6 -- medium with soybean meal. 5 -- 48 hours of growth, staining with Giemsa -- light green; 6 -- 48 hours of growth, staining with Methyl blue.

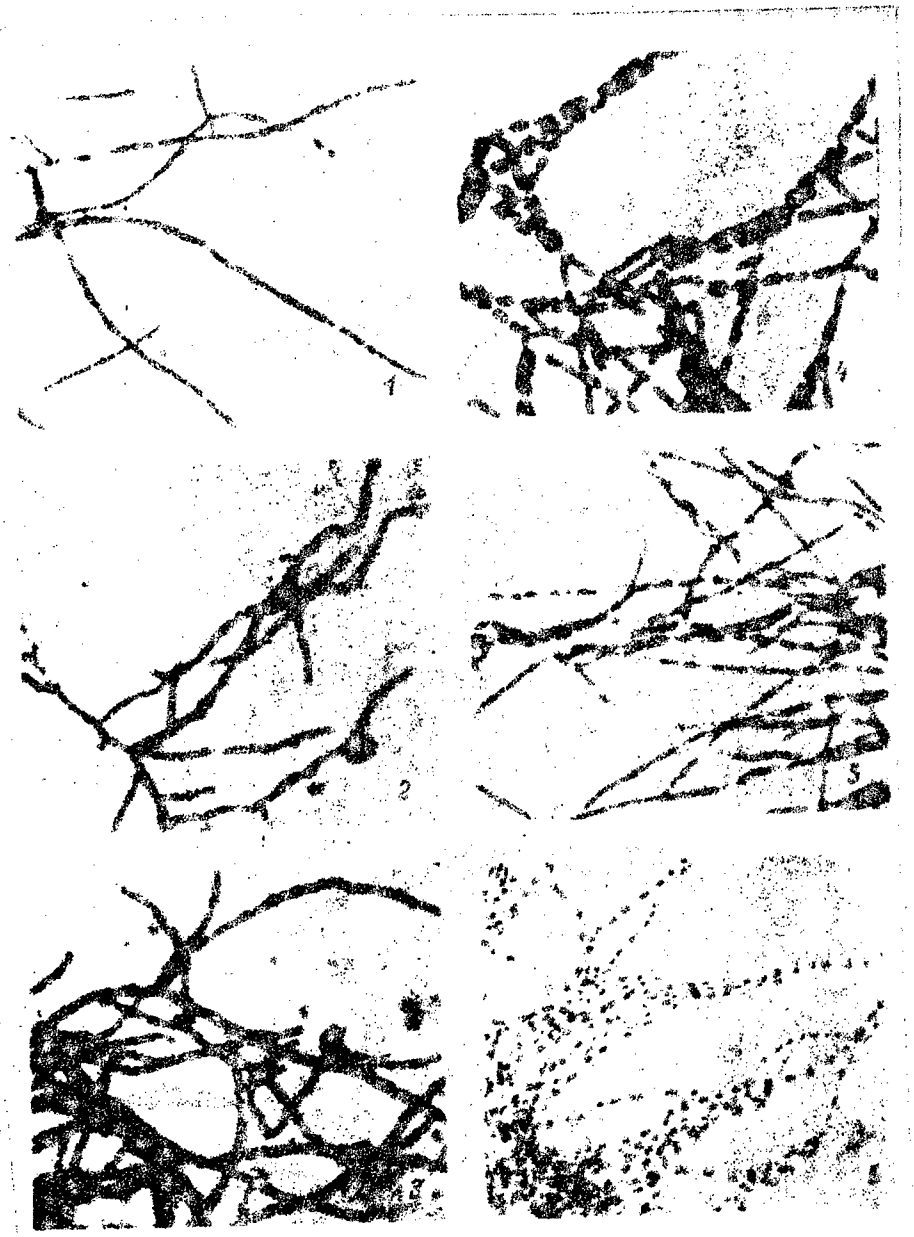

Figure 3. Development of a culture of Actinomyces aureofaciens, strain LS-536, on media with urea and glycocoll. Fixation with Carony's fluid. Microphot., obj. 90x ap. 1.3, eyepiece 10x, magn. 2000x. 1, 2, 3 -- medium with urea: 1 -- 12 hours of growth, staining with methyl blue; 2 -- 48 hours of growth, staining with methyl blue; 3 -- 48 hours of growth, staining with Giemsa -- light green; 4, 5, 6 -- medium with glycocoll: 4 -- 24 hours of growth, staining with methyl blue; 5 -- 48 hours of growth, staining with methyl blue; 6 -- 48 hours of growth, staining according to Fuelgen.

Table 3

Inorganic Phosphorus Content in Media With Different Sources of Nitrogen Nutrition (in mg/%) and Behavior of the Nuclear Substance in Mycelium Cultivated on these Media.

Source of nitrogen	Inorganic phosphorus
Ammonium nitrate (control)	3.80
Soybean meal	4.30
Soybean grist	4.36
Peanut oil cake	4.54
Casein	4.04
Casein hydrolyzate	6.0
Glycocoll	3.80
Soybean grist hydrolyzate	5.70
Leucine	3.80

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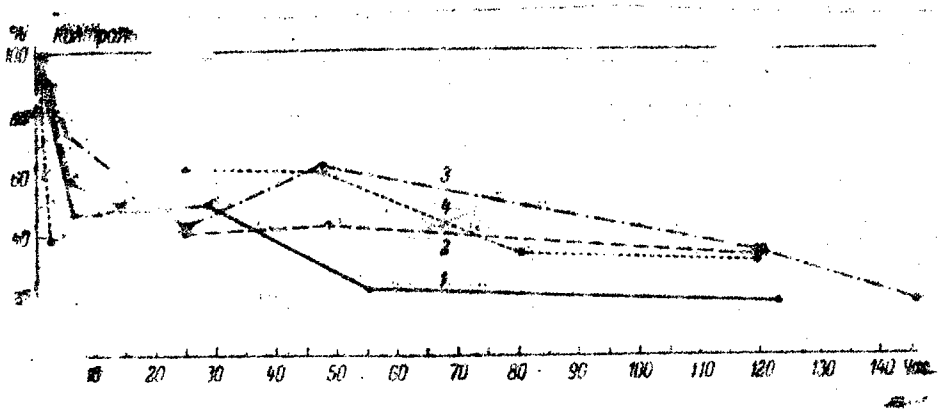

Figure 1. Changes in the resistance of Paramecium Caudatum to 40^{0} when transferred from $15-18^{0}$ to $5-6^{0}$.
Along the axis of ordinates -- survival time at 40^{0} (as % of the control); along the axis of abscissas -- time of transfer of infusorians from 15^{0} to 18^{0} to $5-6^{0}$ (in hours). Clones: 1 -- M', 2 --M, 3--L, 4--8.

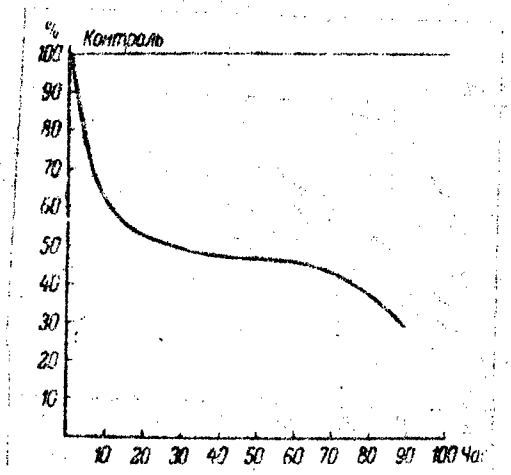

Figure 2. Composite curve (of four clones) showing changes in the resistance of Paramecium caudatum when transferred from 15-18^0 to 5-6^0. Along the axis of ordinates -- survival time at 40^0 (as % of the control); along the axis of abscissas -- time of transfer of infusorians from 15-18^0 to 5-6^0 (in hours).

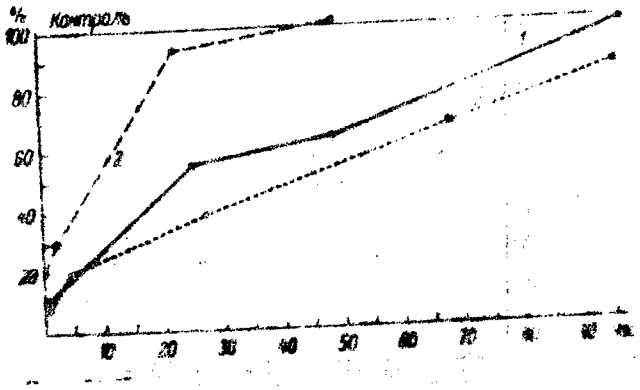

Figure 3. Changes in the heat resistance of Paramecium caudatum to 40° when transferred from 5-6° to 17-18°.
Along the axis of ordinates -- survival time (as % of the control); along the axis of abscissas -- time of transfer of infusorians from 5-6° to 17-18° (in hours). Clones: 1--L, 2--II, 3--5.